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(54) **METHOD FOR PRODUCTION OF L-LYSINE USING METHANOL-UTILIZING BACTERIUM**

(57) Disclosed is a method for production of L-lysine, comprising the steps of cultivating a methanol-utilizing bacterium in a culture medium to produce and accumulate L-lysine in the culture medium and collecting the L-lysine from the culture medium, wherein the methanol-utilizing bacterium carries both of DNA encoding a dihydrodipicolinate synthetase which is desensitized to feedback inhibition by L-lysine and DNA encoding a mutant

LysE protein that can enhance the excretion of L-lysine out of a cell of a methanol-utilizing bacterium when introduced into the methanol-utilizing bacterium and is modified so as to increase the intracellular activities of diaminopimelic acid dehydrogenase, diaminopimelic acid decarboxylase, dihydrodipicolinic acid reductase and aspartate-semialdehyde dehydrogenase.

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Description

Technical Field

5 **[0001]** The present invention relates to microbial industries. More specifically, the present invention relates to a method of producing L-lysine by fermentation, and a microorganism used for the production method.

Background Art

10 **[0002]** L-amino acids such as L-lysine, L-glutamic acid, L-threonine, L-leucine, L-isoleucine, L-valine, and L-phenylalanine are industrially produced by fermentation using microorganisms that belong to the genus *Brevibacterium*, *Corynebacterium*, *Bacillus*, *Escherichia*, *Streptomyces*, *Pseudomonas*, *Arthrobacter*, *Serratia*, *Penicillium*, *Candida* or the like. Strains isolated from nature, or artificial mutants thereof, have been used to improve the productivity of these microorganisms. Moreover, various technologies have been disclosed to increase the ability to produce L-amino acids, 15 such as using a recombinant DNA technology to enhance L-amino acid biosynthetic enzyme activity.

[0003] As for bacteria belonging to the genus *Escherichia*, for example, a method of producing L-lysine by using a strain in which dihydrodipicolinate synthase activity is enhanced has been disclosed (Patent Documents 1 and 2). Further, a method of producing L-lysine using a strain which is transformed with a plasmid containing a DNA encoding dihydrodipicolinate synthase having a mutation to desensitize to feedback inhibition by L-lysine which is derived from a bacterium belonging to the genus *Escherichia*, a DNA encoding aspartokinase that is desensitized to feedback inhibition 20 by L-lysine, a DNA encoding dihydrodipicolinate reductase, and a DNA encoding diaminopimelate dehydrogenase derived from a coryneform bacterium is disclosed in Patent Document 3.

Further, it has been disclosed that production of L-lysine is improved by enhancing an activity of aspartate-semialdehyde dehydrogenase or phosphoenolpyruvate carboxylase and activities of nicotinamide adenine dinucleotide transhydrogenase or aspartase in a bacterium belonging to the genus *Escherichia* having a DNA encoding a dihydrodipicolinate synthase derived from a bacterium belonging to the genus *Escherichia* that has a mutation desensitizing feedback inhibition by L-lysine, a DNA encoding an aspartokinase which is desensitized to feedback inhibition by L-lysine, a DNA encoding a dihydrodipicolinate reductase, and a DNA encoding a diaminopimelate dehydrogenase derived from a coryneform bacterium (Patent Document 4). 25

As for coryneform bacteria, Patent Document 5 discloses that L-lysine productivity can be improved by increasing the intracellular activity of nicotinamide dinucleotide transhydrogenase. 30

Further, a method of producing L-lysine using a strain in which phosphoenolpyruvate carboxylase activity is enhanced and a method of producing L-lysine using a strain in which aspartate-semialdehyde dehydrogenase activity is enhanced are disclosed in Patent Documents 6 and 7, respectively.

35 It has also been reported that by introducing a dihydrodipicolinate synthase gene in addition to a mutant aspartokinase gene having a mutation to desensitize to feedback inhibition, the L-lysine productivity can be improved (Non-patent Document 1).

Moreover, a method of significantly improving the yield of L-lysine without suppressing growth by introducing into a coryneform bacterium a plurality of L-lysine biosynthetic genes, specifically, mutant aspartokinase, dihydrodipicolinate reductase, dihydrodipicolinate synthase, diaminopimelate decarboxylase, and diaminopimelate dehydrogenase in combination has also been disclosed (Patent Document 8). 40

Also, in recent years, the LysE protein and gene encoding it were discovered. The lysE protein functions to export L-lysine specifically to the outside of a microorganism. Also, it has been reported that by increasing the LysE gene in a cell, the L-lysine-producing ability of the cell is increased (Non-patent Document 2 or Patent Document 9).

45 It has also been reported that the production of some L-amino acids can be improved by increasing the expression of the proteins responsible for amino acid export in *Escherichia coli* (Patent Document 10). For example, it has been reported that production of cystine, cysteine, and the like can be improved by enhancing the expression of the ORF306 gene in *Escherichia coli* (Patent Document 11).

[0004] Production of L-amino acids has been considerably increased by breeding of microorganisms and improving production methods as mentioned above. However, in order to respond to further increases in demand in the future, development of more efficient and less expensive methods for production of L-amino acids is still desirable. 50

[0005] Methanol is known to be a raw material for fermentation and is available in large quantities at a low cost. Methods of producing L-amino acids by fermentation using methanol are known, and include those using microorganisms that belong to the genus *Achromobacter* or *Pseudomonas* (Patent Document 12), *Prolaminobacter* (Patent Document 13), *Protaminobacter* or *Methanomonas* (Patent Document 14), *Microcycylus* (Patent Document 15), *Methylobacillus* (Patent Document 16), *Bacillus* (Patent Document 17) and the like. 55

The inventors of the present invention have developed methods of producing L-amino acids by breeding bacteria belonging to the genus *Methylophilus* using artificial mutagenesis and a recombinant DNA technology to enhance activities

of dihydrodipicolinate synthase and aspartokinase (Patent Document 18). The inventors of the present invention showed that the amino acid export process is a big hurdle in amino acid production by fermentation of methanol using a methanol-assimilating bacterium. In order to solve the problems, the inventors obtained a mutant that exhibits L-lysine-exporting activity in a methanol-assimilating bacterium from a LysE protein that is isolated from a bacterium belonging to the genus *Corynebacterium* and involved in export of L-lysine. The inventors of the present invention disclosed that L-lysine can be efficiently produced by: breeding bacteria belonging to the genus *Methylophilus* using artificial mutagenesis, a recombinant DNA technology, and the mutant L-lysine exporter, and the inventors have developed a method of producing an L-amino acid using a bacterium belonging to the genus *Methylophilus* (Patent Document 18 or 19).

[0006] However, for production of L-amino acids by fermentation of methanol using a methanol-assimilating bacterium, there has been no report of significant improvements in L-lysine yield by enhancing expression of an L-lysine export gene in combination with a plurality of L-lysine biosynthetic genes.

[0007] It is known that enhancement of either *ddh* or *lysA* in a coryneform bacterium can improve the amount of produced Lys. It has also been disclosed that enhancement of *ddh* and *lysA* in combination can improve the Lys production rate and the amount of produced Lys.

However, enhancement of either of these genes in a bacterium belonging to the genus *Methylophilus* could not increase, and rather slightly decreased the amount of produced L-lysine.

Patent Document 1: JP 56-018596 A

Patent Document 2: US Patent 4,346,170

Patent Document 3: WO 95/16042 (US 6,040,160)

Patent Document 4: WO 01/53459 (EP 1,253,195 A)

Patent Document 5: WO 95/11985 (US 5,830,716)

Patent Document 6: JP 60-87788 A

Patent Document 7: JP 06-102028 B

Patent Document 8: WO 96/40934 (EP 841,395)

Patent Document 9: WO 97/23597 (US 6,858,406)

Patent Document 10: JP 2000-189180 A (EP 1,016,710 A)

Patent Document 11: EP 885,962 B

Patent Document 12: JP 45-25273 A

Patent Document 13: JP 49-125590 B

Patent Document 14: JP 50-25790 A

Patent Document 15: JP 52-18886 A

Patent Document 16: JP 04-91793 A

Patent Document 17: JP 03-505284 A

Patent Document 18: WO 00/61723 (EP 1,188,822 A)

Patent Document 19: JP 2004-166594 A (US 2005-003495 A)

Non-patent Document 1: Applied and Environmental Microbiology 57(6), 1746-1752(1991)

Non-patent Document 2: Molecular Microbiology 22:815-826 (1996)

Disclosure of the Invention

[0008] It is an object of the present invention to provide a method of efficiently producing L-lysine using methanol. Methanol is abundantly and inexpensively available.

[0009] The inventors of the present invention assiduously studied in order to achieve the aforementioned object. As a result, the inventors have succeeded in remarkable improvement in L-lysine yield by enhancing a gene encoding an L-lysine exporter in combination with a plurality of L-lysine biosynthetic genes. That is, the inventors found the L-lysine yield is improved by enhancing expression of the diaminopimelate decarboxylase gene and the diaminopimelate dehydrogenase gene in addition to a DNA encoding a mutant LysE protein and a DNA encoding dihydrodipicolinate synthase which is desensitized to feedback inhibition by L-lysine in a bacterium belonging to the genus *Methylophilus*, which was never achieved by enhancing expression of only one of the genes. Moreover, the inventors found that the L-lysine-producing ability of a bacterium belonging to the genus *Methylophilus* is further improved by enhancing expression of the dihydrodipicolinate reductase gene and the aspartate-semialdehyde dehydrogenase gene in addition to the above-mentioned genes, and that the ability is significantly improved by enhancing an aspartokinase gene.

Meanwhile, the inventors have achieved the construction of a very stable strain by enhancing the expression of the biosynthetic genes with a plasmid together with incorporating multiple copies of the genes into chromosome of a methanol-assimilating bacterium. The inventors further enhanced gene expression with a plasmid in combination, to thereby obtain a methanol-assimilating bacterium having a significantly improved L-lysine yield.

Based on these findings, the inventors have accomplished the present invention.

[0010] That is, the present invention provides the followings.

(1) A methanol-assimilating bacterium, which comprises:

a DNA encoding a dihydrodipicolinate synthase which is desensitized to feedback inhibition by L-lysine; and
a DNA encoding a mutant LysE protein that promotes export of L-lysine to the outside of a bacterial cell when it is introduced into a methanol-assimilating bacterium, wherein said bacterium is modified to enhance intracellular activities of diaminopimelate dehydrogenase, diaminopimelate decarboxylase, dihydrodipicolinate reductase, and aspartate-semialdehyde dehydrogenase.

(2) The methanol-assimilating bacterium according to (1), further comprising:

a DNA encoding an aspartokinase which is desensitized to feedback inhibition by L-lysine.

(3) The methanol-assimilating bacterium according to (1) or (2), wherein said bacterium is introduced with DNAs encoding the enzymes or the mutant LysE protein into chromosomal DNA and/or transformed with a plasmid(s) carrying DNAs encoding the enzymes or the mutant LysE protein.

(4) The methanol-assimilating bacterium according to (3), wherein the DNA encoding dihydrodipicolinate synthase, the DNA encoding dihydrodipicolinate reductase, the DNA encoding aspartate-semialdehyde dehydrogenase, and the DNA encoding aspartokinase are derived from a bacterium belonging to the genus *Escherichia*, the DNA encoding diaminopimelate decarboxylase is derived from a bacterium belonging to the genus *Methylophilus*, and the DNA encoding diaminopimelate dehydrogenase and the DNA encoding mutant LysE are derived from a bacterium belonging to the genus *Brevibacterium*.

(5) The methanol-assimilating bacterium according to any one of (1) to (4), wherein said bacterium belongs to the genus *Methylophilus*.

(6) The methanol-assimilating bacterium according to any one of (1) to (5), wherein said bacterium is *Methylophilus methylotrophus* AJ110196 strain (FERM BP-10434).

(7) A method of producing L-lysine, comprising culturing the methanol-assimilating bacterium according to any one of (1) to (6) in a medium to produce and accumulate L-lysine in the medium or bacterial cells; and
collecting L-lysine from the medium or bacterial cells.

(8) The method of producing L-lysine according to (7), wherein the main carbon source of the medium is methanol.

(9) *Methylophilus methylotrophus* AJ110196 strain (FERM BP-10434).

Brief Description of the Drawings

[0011]

Fig. 1 is an illustration showing the construction of plasmid pM12.

Fig. 2 is an illustration showing the construction of plasmid pMIV5.

Fig. 3 is an illustration showing the construction of plasmid pMIV-FRTKmFRT.

Fig. 4 is an illustration showing the construction of plasmid pAET7.

Fig. 5 is an illustration showing the construction of plasmid pFLP31.

Description of the Preferred Embodiments

[0012] Hereinafter, the present invention will be described in detail.

[0013] The methanol-assimilating bacterium of the present invention is a methanol-assimilating bacterium which carries a DNA encoding a dihydrodipicolinate synthase that is desensitized to feedback inhibition by L-lysine and a DNA encoding a mutant LysE that promotes the export of L-lysine to the outside of a bacterial cell when the DNA is introduced into the methanol-assimilating bacterium, and has been further modified to enhance the intracellular activities of diaminopimelate dehydrogenase, diaminopimelate decarboxylase, dihydrodipicolinate reductase, and aspartate-semialdehyde dehydrogenase.

[0014] In the present invention, the methanol-assimilating bacterium is a bacterium that can grow in a medium containing methanol as the main carbon source and includes a bacterium belonging to the genus *Methylophilus* and the like. Specific examples thereof include a bacterium belonging to the genus *Methylophilus* such as *Methylophilus methylotrophus*. Examples of *Methylophilus methylotrophus* include the AS 1 strain (NCIMB 10515). *Methylophilus methylotrophus* AS 1 strain (NCIMB 10515) is available from the National Collections of Industrial and Marine Bacteria (Address: NCIMB Ltd., Torry Research Station 135, Abbey Road, Aberdeen AB9 8DG, United Kingdom).

<DNA encoding dihydrodipicolinate synthase which is desensitized to feedback inhibition by L-lysine>

[0015] The DNA encoding a dihydrodipicolinate synthase which is desensitized to feedback inhibition by L-lysine (hereinafter, also referred to as dapA* gene) is not particularly limited, however, it is preferably, for example, a DNA encoding a dihydrodipicolinate synthase derived from a bacterium belonging to the genus *Escherichia* and having a mutation to desensitize to feedback inhibition by L-lysine.

Examples of the DNA encoding a wild-type dihydrodipicolinate synthase derived from a bacterium belonging to the genus *Escherichia* include a DNA encoding the amino acid sequence of SEQ ID NO: 41. Examples of the mutation to desensitize to feedback inhibition by L-lysine include a mutation that replaces the histidine residue at position 118 in the amino acid sequence of SEQ ID NO: 41 with a tyrosine residue (H118Y mutation). Therefore, examples of the dapA* gene include a DNA encoding the amino acid sequence of SEQ ID NO: 41 in which the histidine residue at position 118 is replaced with a tyrosine residue.

The dapA* gene may be a DNA encoding a protein having at least 80%, preferably at least 90%, more preferably at least 95%, particularly preferably at least 98% homology to the entire amino acid sequence of SEQ ID NO: 41, having H118Y mutation, and having dihydrodipicolinate synthase activity.

Moreover, the dapA* gene may be a DNA encoding a protein having an amino acid sequence of SEQ ID NO: 41 including substitution, deletion, insertion, addition, or the like of one or several amino acids so long as the protein has H118Y mutation and the dihydrodipicolinate synthase activity is not impaired.

Although the number of the "several" amino acids referred herein may differ depending on positions in the three-dimensional structure of the proteins or types of amino acid residues, it is specifically 2 to 20, preferably 2 to 10, and more preferably 2 to 5. The above-mentioned substitution of amino acids is preferably a conservative substitution. Examples of conservative substitutions include: substitution of ser or thr for ala; substitution of gln, his, or lys for arg; substitution of glu, gln, lys, his, or asp for asn; substitution of asn, glu, or gln for asp; substitution of ser or ala for cys; substitution of asn, glu, lys, his, asp, or arg for gln; substitution of gly, asn, gln, lys, or asp for glu; substitution of pro for gly; substitution of asn, lys, gln, arg, or tyr for his; substitution of leu, met, val, or phe for ile; substitution of ile, met, val, or phe for leu; substitution of asn, glu, gln, his, or arg for lys; substitution of ile, leu, val or phe for met; substitution of trp, tyr, met, ile, or leu for phe; substitution of thr or ala for ser; substitution of ser or ala for thr; substitution of phe or tyr for trp; substitution of his, phe, or trp for tyr; and substitution of met, ile, or leu for val. The above-mentioned amino acid substitutions, deletions, insertions, additions, inversions, or the like may be the result of a naturally-occurring mutation (mutant or variant) due to an individual difference, or a difference of bacterial species harboring the dihydrodipicolinate synthase gene.

The dapA* gene may be a DNA which is able to hybridize with a complementary strand of the nucleotide sequence of SEQ ID NO: 40 or a probe that can be prepared from the sequence under stringent conditions so long as the gene encodes a protein having the H118Y mutation and having dihydrodipicolinate synthase activity. The term "stringent conditions" refers to conditions where a so-called specific hybrid is formed and a non-specific hybrid is not formed. It is difficult to clearly define the conditions with a numerical value, but examples thereof include conditions corresponding to salt concentration and temperature of washing conditions of typical Southern hybridization, e.g., washing at 60°C with 1 × SSC and 0.1% SDS, preferably at 60°C with 0.1 × SSC and 0.1% SDS, and more preferably at 68°C with 0.1 × SSC and 0.1% SDS, once or preferably twice or three times.

The dapA* gene may be obtained by site-specific mutagenesis, or from the RSFD80 plasmid as described below. It is known that the wild-type dihydrodipicolinate synthase derived from coryneform bacteria is not subject to feedback inhibition by L-lysine (J Gen Microbiol. 1988 Dec;134 (12) :3221-9.). Therefore, it is not always necessary that the DNA encoding a dihydrodipicolinate synthase which is desensitized to feedback inhibition by L-lysine, to be used in the present invention, be a DNA encoding a mutant dihydrodipicolinate synthase.

<DNA encoding mutant LysE that promotes export of L-lysine to the outside of a bacterial cell when the DNA is introduced into the methanol-assimilating bacterium>

[0016] Examples of the DNA encoding mutant LysE that promotes export of L-lysine to the outside of a bacterial cell when the DNA is introduced into the methanol-assimilating bacterium include the LysE24 gene (US 2003-0124687), lysE56 gene (US 2004-0146974), and lysE24m5 (WO 2006/059715).

The expression "promoting export of L-lysine to the outside of a bacterial cell" means that when a methanol-assimilating bacterium containing the DNA is cultured in a medium, the amount of L-lysine exported into the medium increases as compared with the methanol-assimilating bacterium not containing the DNA. An increase in export of the L-lysine to the outside of the cell occurs when there is an increase in L-lysine accumulation in the medium during the culture of the methanol-assimilating bacterium containing the DNA as compared with the accumulation when the methanol-assimilating bacterium not containing the DNA is cultured.

The DNA encoding mutant LysE that promotes export of L-lysine to the outside of a bacterial cell when the DNA is

introduced into the methanol-assimilating bacterium is preferably, but is not limited to, a DNA encoding the LysE protein derived from a bacterium belonging to the genus *Brevibacterium* and having a mutation for promoting export of L-lysine to the outside of a bacterial cell when the DNA is introduced into the methanol-assimilating bacterium, and examples thereof include the LysE24 gene (US 2003-0124687), lysE56 gene (US 2004-0146974), and lysE24m5 (WO 2006/059715).

(1) lysE24 gene

[0017] The lysE24 gene is a DNA encoding a mutant of a protein having a loop region and six hydrophobic helices that is involved in the export of L-lysine to the outside of a bacterial cell (wild-type lysE protein: SEQ ID NO: 55), wherein the mutant lysE protein does not have the above-mentioned loop region and promotes export of L-lysine, L-arginine, or both to the outside of a bacterial cell when the DNA is introduced into the methanol-assimilating bacterium. Examples of the LysE24 gene include the LysE24 gene described in JP 2004-166594 A (US 2005-003495).

An example thereof includes a DNA encoding the protein of SEQ ID NO: 51. In addition, the protein may be a protein having not less than 80%, preferably not less than 90%, more preferably not less than 95%, particularly preferably not less than 98% homology to the entire amino acid sequence of SEQ ID NO: 51 so long as the gene can promote export of L-lysine to the outside of a bacterial cell when it is introduced into a methanol-assimilating bacterium.

Moreover, the gene may be a DNA encoding a protein having a sequence of SEQ ID NO: 51 including substitutions, deletions, insertions, additions, or the like of one or several amino acids so long as the activity for promoting export of L-lysine to the outside of a bacterial cell is not impaired.

Moreover, the lysE24 gene may be a DNA which is able to hybridize with a complementary strand of the nucleotide sequence of SEQ ID NO: 50 or a probe that can be prepared from the nucleotide sequence under stringent conditions so long as the gene encodes a protein having the activity for promoting export of L-lysine to the outside of a bacterial cell. The definitions of the terms "several" and "stringent conditions" and preferable amino acid substitutions are the same as described above.

The lysE24 gene can be obtained from, for example, the pRSlysE24 plasmid described in JP 2004-166594 A (US 2005-003495). *E. coli* JM109 strain transformed with pRSlysE24 was designated as AJ13830, and deposited at National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary on June 4, 2001 and given the accession number FERM P-18369. Then, the deposit was transferred to an international deposition under the provisions of the Budapest Treaty on May 13, 2002 and given the accession number FERM BP-8040.

(2) lysE56 gene

[0018] An example of the lysE56 gene includes a gene encoding a protein having an amino acid sequence of SEQ ID NO: 55 in which at least the glycine residue at position 56 is replaced with another amino acid residue (US 2004-0146974). The gene may also be a gene encoding a protein having an amino acid sequence of SEQ ID NO: 55 having not less than 80%, preferably not less than 90%, more preferably not less than 95%, particularly preferably not less than 98% homology to SEQ ID NO: 55, in which the glycine residue at position 56 is replaced with another amino acid residue, so long as the gene can promote export of L-lysine to the outside of a bacterial cell when it is introduced into a methanol-assimilating bacterium.

(3) lysE24m5 gene

[0019] This gene is a DNA having a nucleotide sequence of the lysE24 gene, which has been modified so that all the three kinds of reading frames include stop codons, and promotes export of L-lysine, L-arginine, or both to the outside of a bacterial cell by a methanol-assimilating bacterium when the DNA is introduced into the bacterium. Specific examples thereof include the DNA having the nucleotide sequence of SEQ ID NO: 56 (WO 2006/059715). The gene may be a DNA which is able to hybridize with a complementary strand of the nucleotide sequence of SEQ ID NO: 56 or a probe that can be prepared from the nucleotide sequence under stringent conditions so long as the gene encodes a protein having activity for promoting export of L-lysine to the outside of a bacterial cell.

<ddh gene>

[0020] The diaminopimelate dehydrogenase activity can be enhanced using a gene encoding a diaminopimelate dehydrogenase (hereinafter, also referred to as ddh gene).

An example of the ddh gene includes, but is not limited to, a DNA encoding a diaminopimelate dehydrogenase derived from a coryneform bacterium (SEQ ID NO: 53).

The ddh gene may be a DNA encoding a protein having not less than 80%, preferably not less than 90%, more preferably

not less than 95%, particularly preferably not less than 98% homology to the entire amino acid sequence of SEQ ID NO: 53 and having the diaminopimelate dehydrogenase activity.

The *ddh* gene may also be a DNA encoding a protein having a sequence of SEQ ID NO: 53 including substitutions, deletions, insertions, additions, or the like of one or several amino acids so long as diaminopimelate dehydrogenase activity is not impaired.

Moreover, the *ddh* gene may be a DNA which is able to hybridize with a complementary strand of the nucleotide sequence of SEQ ID NO: 52 or a probe that can be prepared from the nucleotide sequence under stringent conditions so long as the gene encodes a protein having the diaminopimelate dehydrogenase activity.

The definitions of the terms "several" and "stringent conditions" and preferable amino acid substitution are the same as described above.

The *ddh* gene of a coryneform bacterium can be obtained by amplification through PCR using two oligonucleotide primers (for example, SEQ ID NOS: 11 and 12 described in WO/9516042) which are prepared based on the known nucleotide sequence of *ddh* from *Corynebacterium glutamicum* (Ishino, S. et al., Nucleic Acid Res., 15, 3917 (1987)) and using the chromosomal DNA of *Brevibacterium lactofermentum* or *Corynebacterium glutamicum* as the template.

<lysA gene>

[0021] The diaminopimelate decarboxylase activity can be enhanced using the diaminopimelate decarboxylase gene (hereinafter, also referred to as *lysA* gene). An example of the *lysA* gene includes, but is not limited to, a DNA encoding diaminopimelate decarboxylase derived from a bacterium belonging to the genus *Methylophilus* (SEQ ID NO: 49).

The *lysA* gene may be a DNA encoding a protein having not less than 80%, preferably not less than 90%, more preferably not less than 95%, particularly preferably not less than 98% homology to the entire amino acid sequence of SEQ ID NO: 49 and having the diaminopimelate decarboxylase activity.

The *lysA* gene may also be a DNA encoding a protein having a sequence of SEQ ID NO: 49 including substitutions, deletions, insertions, additions, or the like of one or several amino acids so long as diaminopimelate decarboxylase activity is not impaired.

Moreover, the *lysA* gene may be a DNA which is able to hybridize with a complementary strand of the nucleotide sequence of SEQ ID NO: 48 or a probe that can be prepared from the nucleotide sequence under stringent conditions so long as the gene encodes a protein having diaminopimelate decarboxylase activity.

The definitions of the terms "several" and "stringent conditions" and preferable amino acid substitutions are the same as described above.

The *lysA* gene of *Methylophilus methylotrophus* can be obtained by PCR using two oligonucleotide primers prepared based on the known sequence and using the chromosomal DNA of *Methylophilus methylotrophus* as the template.

<dapB gene>

[0022] The dihydrodipicolinate reductase activity can be enhanced using a gene encoding dihydrodipicolinate reductase (hereinafter, also referred to as *dapB* gene). An example of the *dapB* gene includes, but is not limited to, a DNA encoding a dihydrodipicolinate reductase derived from a bacterium belonging to the genus *Escherichia* (SEQ ID NO: 43).

The *dapB* gene may be a DNA encoding a protein having not less than 80%, preferably not less than 90%, more preferably not less than 95%, particularly preferably not less than 98% homology to the entire amino acid sequence of SEQ ID NO: 43 and having the dihydrodipicolinate reductase activity.

The *dapB* gene may also be a DNA encoding a protein having a sequence of SEQ ID NO: 43 including substitutions, deletions, insertions, additions, or the like of one or several amino acids so long as the dihydrodipicolinate reductase activity is not impaired.

Moreover, the *dapB* gene may be a DNA which is able to hybridize with a complementary strand of the nucleotide sequence of SEQ ID NO: 42 or a probe that can be prepared from the nucleotide sequence under stringent conditions so long as the gene encodes a protein having the dihydrodipicolinate reductase activity.

The definitions of the terms "several" and "stringent conditions" and preferable amino acid substitutions are the same as described above.

The dihydrodipicolinate reductase gene (*dapB*) can be amplified by PCR using two oligonucleotide primers prepared based on the known nucleotide sequence and using a chromosomal DNA of *E. coli* as the template.

<asd gene>

[0023] The aspartate-semialdehyde dehydrogenase activity can be enhanced using a gene encoding aspartate-semialdehyde dehydrogenase (hereinafter, also referred to as *asd* gene). An example of the *asd* gene includes, but is not limited to, a DNA encoding a aspartate-semialdehyde dehydrogenase derived from a bacterium belonging to the genus

Escherichia (SEQ ID NO: 45).

The *asd* gene may be a DNA encoding a protein having not less than 80%, preferably not less than 90%, more preferably not less than 95%, particularly preferably not less than 98% homology to the entire amino acid sequence of SEQ ID NO: 45 and having the aspartate-semialdehyde dehydrogenase activity.

The *asd* gene may also be a DNA encoding a protein having a sequence of SEQ ID NO: 45 including substitutions, deletions, insertions, additions, or the like of one or several amino acids so long as the aspartate-semialdehyde dehydrogenase activity is not impaired.

Moreover, the *asd* gene may be a DNA which is able to hybridize with a complementary strand of the nucleotide sequence of SEQ ID NO: 44 or a probe that can be prepared from the nucleotide sequence under stringent conditions so long as the gene encodes a protein having the aspartate-semialdehyde dehydrogenase activity.

The definitions of the terms "several" and "stringent conditions" and preferable amino acid substitutions are the same as described above.

The aspartate-semialdehyde dehydrogenase gene (*asd*) can be amplified by PCR using two oligonucleotide primers prepared based on the known nucleotide sequence and using the chromosomal DNA of *E. coli* as the template.

<lysC* gene>

[0024] The methanol-assimilating bacterium of the present invention may further comprise a DNA encoding aspartokinase that is desensitized to feedback inhibition by L-lysine.

Examples of the DNA encoding an aspartokinase that is desensitized to feedback inhibition by L-lysine (hereinafter, also referred to as lysC* gene) preferably include, but are not limited to, a DNA encoding an aspartokinase derived from a bacterium belonging to the genus *Escherichia* and having a mutation to desensitize to feedback inhibition by L-lysine.

An example of the DNA encoding a wild-type aspartokinase derived from a bacterium belonging to the genus *Escherichia* includes a DNA encoding the amino acid sequence of SEQ ID NO: 47. An example of the mutation to desensitize to feedback inhibition by L-lysine includes a mutation that replaces the threonine residue at position 352 with an isoleucine residue in the amino acid sequence of SEQ ID NO: 47 (T352I mutation). Therefore, an example of the lysC* gene includes a DNA encoding the amino acid sequence of SEQ ID NO: 47 in which the threonine residue at position 352 is replaced with an isoleucine residue.

The lysC* gene may be a DNA encoding a protein having not less than 80%, preferably not less than 90%, more preferably not less than 95%, particularly preferably not less than 98% homology to the entire amino acid sequence of SEQ ID NO: 47, having the T352I mutation, and having the aspartokinase activity.

The lysC* gene may also be a DNA encoding a protein having a sequence of SEQ ID NO: 47 including substitutions, deletions, insertions, additions, or the like of one or several amino acids so long as it has the T352I mutation and the aspartokinase activity is not impaired.

Moreover, the lysC* gene may be a DNA which is able to hybridize with a complementary strand of the nucleotide sequence of SEQ ID NO: 46 or a probe that can be prepared from the nucleotide sequence under stringent conditions so long as the gene has the T352I mutation and encodes a protein having the aspartokinase activity.

The definitions of the terms "several" and "stringent conditions" and preferable amino acid substitutions are the same as described above.

The lysC* gene may be obtained by site-specific mutagenesis, or from the RSFD80 plasmid as described below.

It is not always necessary that the DNA encoding an aspartokinase that is desensitized to feedback inhibition by L-lysine, to be used in the present invention, be a DNA encoding a mutant aspartokinase. That is, a wild-type protein may be used so long as the protein is not subject to feedback inhibition by L-lysine.

[0025] In this description, the phrase "intracellular activity is enhanced" means that the intracellular enzymatic activity is increased as compared with a wild-type strain (for example, *M. methylotrophus* AS 1 strain) or a parent strain (strain in which intracellular activities of all of the enzymes specified in the present invention are not enhanced), and also means that a bacterium has an enzymatic activity that is not possessed by a wild-type strain or a parent strain. Specifically, although a bacterium belonging to the genus *Methylophilus* does not have *dapA** gene, *lysE* gene, and *ddh* gene encoding diaminodipicolinate dehydrogenase, if intracellular enzymatic activities are detected or one or more copies of the *dapA** gene, mutant *lysE* gene, and *ddh* gene is amplified, such a bacterium is considered to have increased intracellular activities.

Examples of a procedure for enhancing an intracellular activity include, but are not limited to, the following procedures and combinations thereof.

- (1) increasing the copy number by transformation with a plasmid carrying a DNA encoding each protein.
- (2) increasing the copy number by incorporating a DNA encoding each protein into a chromosome.
- (3) increasing the expression amount by modifying a promoter sequence of a gene encoding each protein.

[0026] Examples of a procedure for introducing the dapA* gene, mutant lysE gene, and ddh gene include, but are not limited to, the following procedures.

- (1) increasing the copy number by transformation with a plasmid carrying a DNA encoding each protein.
- (2) increasing the copy number by incorporating a DNA encoding each protein into the chromosome.

In addition, these procedures may be combined with modification of a promoter sequence.

[0027] The increase in an enzymatic activity can be confirmed by: a method of Yugari, Y et al. (Biochim. Biophys. Acta 62 612-614 1962) for a dihydrodipicolinate synthase (E4.2.1.52.); a method of Misono, H et al. (J. Biol. Chem. 255, 10599-10605 1980) for a diaminodipicolinate dehydrogenase (EC: 1.4.1.16); a method of White, P.J et al. ((1965) Biochem, J, 96,75-84) for a diaminopimelate decarboxylase (EC4.1.1.20); a method of Kimura, H et al. (J. Biochem, 77, 405-413, 1975) for a dihydrodipicolinate reductase (EC: 1.3.1.26); a method of Black, S et al. (J. Biol Chem. 213 39-50 1955) for an aspartate-semialdehyde reductase (EC 1.2.1.11); or a method of Truffa-Bachi et al. (The Enzymes (3rd ed.) 8,509-553) for an aspartokinase (EC:2.7.2.4). A control strain to be used for these methods may be *Methylophilus methylotrophus* AS 1 strain (NCIMB 10515).

[0028] Enhancement of an enzymatic activity can be confirmed by comparing the amount of m-RNA for a gene encoding each enzyme with that of a wild-type or unmodified strain. Examples of methods to confirm the expression amount include Northern hybridization and RT-PCR (Molecular Cloning (Cold spring Harbor Laboratory Press, Cold spring Harbor (USA), 2001)). The activity or expression amount is increased as compared to that of a wild-type or unmodified strain, and it is desirably increased, for example, not less than 1.5-fold, preferably not less than 2-fold, more preferably not less than 3-fold, further more preferably not less than 5-fold, further more preferably not less than 10-fold as compared to that of a wild-type or unmodified strain. In particular, the enzymatic activity of a dihydrodipicolinate synthase is increased preferably not less than 10-fold, more preferably not less than 15-fold, and still more preferably not less than 20-fold.

[0029] The increase in the copy number can be confirmed by Southern hybridization using a probe complementary to a target gene. The gene is amplified preferably 3 or more copies, more preferably 5 or more copies, and still more preferably 10 or more copies. In particular, the dapB gene, lysA gene, ddh gene, or asd gene introduced is preferably 2 or more copies, and more preferably 3 or more copies; the lysC* gene introduced is preferably 3 or more copies and more preferably 4 or more copies; and the dapA gene or mutant lysE gene introduced is preferably 10 or more copies, preferably 12 or more copies, and still more preferably 15 or more copies.

[0030] The methanol-assimilating bacterium of the present invention has an L-lysine producing ability, which has been imparted by the above-mentioned modification (enhancement of expression of each gene: mutant lysE, dapA*, lysA, ddh, dapB, asd, and (lysC*)). The term "L-lysine producing ability" as used herein refers to an ability to accumulate L-lysine in a medium to such a degree that L-lysine can be collected from the medium when the methanol-assimilating bacterium of the present invention is cultured in the medium.

[0031] The methanol-assimilating bacterium of the present invention may be a mutant strain that has mutations such as an auxotrophic mutation, an analogue resistant mutation, and a metabolic regulation mutation and has been modified as described above.

For example, the bacterium may be a mutant strain which is auxotrophic for L-homoserine, or L-threonine and L-methionine (JP 48-28078 B and JP 56-6499 B), a mutant strain that is auxotrophic for inositol or acetic acid (JP 55-9784 A and JP 56-8692 A), or a mutant strain that is resistant to oxalysine, lysine hydroxamate, S-(2-aminoethyl)-cysteine, γ -methyllysine, α -chlorocaprolactam, DL- α -amino- ϵ -caprolactam, α -amino-lauryllactam, aspartic acid analogue, sulfa drug, quinoid or N-lauroylleucine, and has been modified as described above.

The mutations such as an auxotrophic mutation, an analogue resistant mutation, and a metabolic regulation mutation may be introduced after the modifications as described above (gene amplification).

[0032] A method of enhancing expression of a dihydrodipicolinate synthase gene (dapA* gene) and an aspartokinase gene (lysC* gene), both of which are not subject to feedback inhibition by L-lysine, will be described. The expression of mutant lysE gene, dapB gene, lysA gene, ddh gene, and asd gene can also be enhanced by the same method.

The expression of the dapA* gene and lysC* gene can be enhanced by transforming a host such as a bacterium belonging to the genus *Methylophilus* with a recombinant DNA prepared by ligating such DNA fragments with a vector that functions in the *Methylophilus* bacterium and preferably a multi-copy type vector. The increase in copy number of the genes results in an enhancement of the intracellular activities of enzymes encoded by target genes of dihydrodipicolinate synthase and aspartokinase. Hereinafter, dihydrodipicolinate synthase, aspartokinase, and aspartokinase III are also referred to as DDPS, AK and AKIII, respectively, in some cases.

[0033] The genes encoding DDPS and AK may be derived from any microorganism so long as the chosen microorganism carries a DNA expressing DDPS and AK activity in a bacterium belonging to the genus *Methylobacillus*. Such microorganisms may be wild-type strains, or mutant strains derived therefrom. Specifically, examples of such microorganisms include *E.coli* (*Escherichia coli*) K-12 strain and *Methylophilus methylotrophus* AS1 strain (NCIMB10515). Since nucleotide sequences for the genes encoding DDPS (Richaud, F. et al., J. Bacteriol., 297 (1986)) and AKIII

(Cassan, M., Parsot, C., Cohen, G. N. and Patte, J. C., J. Biol. Chem., 261, 1052 (1986)) are known, these genes can be obtained by PCR using primers synthesized based on the nucleotide sequences of these genes, and using chromosomal DNA of microorganism such as *E.coli* K-12 as a template. Hereinafter, description will be given using dapA and lysC derived from *E.coli* as examples, but the genes to be used in the present invention are not limited thereto.

[0034] The DDPS and AK used for the present invention are not subject to feedback inhibition by L-lysine. It is known that wild-type DDPS derived from *E.coli* is subject to feedback inhibition by L-lysine, and that wild-type AKIII derived from *E.coli* is subject to suppression and feedback inhibition by L-lysine. Therefore, in order to introduce the genes into a bacterium belonging to the genus *Methylophilus*, it is preferable to introduce a mutation that eliminates the feedback inhibition by L-lysine into the genes.

However, it is not always necessary that the DDPS and AK genes used for the present invention be mutated because, for example, DDPS derived from a bacterium belonging to the genus *Corynebacterium* is not subject to feedback inhibition by L-lysine.

[0035] The dapA* and the lysC* which respectively encode DDPS and AK that are desensitized to feedback inhibition by L-lysine can be obtained by PCR using a plasmid containing the genes as a template and using two oligonucleotide primers prepared based on known nucleotide sequences.

The broad-host-range plasmid RSFD80 is known as the plasmid containing the dapA* and lysC* (WO 95/16042). The *E.coli* JM109 strain transformed with this plasmid was designated as AJ 12396, and deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (currently, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary) on October 28, 1993 and given the accession number FERM P-13936. Then, the deposit was transferred to an international deposition under the provisions of the Budapest Treaty on November 1, 1994 and given the accession number FERM BP-4859. RSFD80 can be obtained from the AJ12396 strain by a known method.

[0036] The dapA* contained in RSFD80 has the nucleotide sequence of the wild-type dapA gene (SEQ ID NO: 40) in which C at nucleotide number 597 is replaced with T, and thereby mutant DDPS encoded by the gene has the amino acid sequence of SEQ ID NO: 41 in which the histidine residue at position 118 is replaced with a tyrosine residue. The lysC* contained in RSFD80 has the nucleotide sequence of the wild-type lysC gene (SEQ ID NO: 46) in which C at nucleotide number 1638 is replaced with T, and thereby mutant AKIII encoded by the gene has the amino acid sequence of SEQ ID NO: 47 in which the threonine residue at position 352 is replaced with an isoleucine residue.

[0037] The plasmid used for gene cloning may be any plasmid so long as it can replicate in microorganisms such as bacteria belonging to the genus *Escherichia*. Specifically, examples of such plasmids include pBR322, pTWV228, pMW119, and pUC19.

[0038] Vectors that function in bacteria belonging to the genus *Methylophilus* include, for example, a plasmid that can autonomously replicate in bacteria belonging to the genus *Methylophilus*. Specifically, examples thereof include RSF1010, which is a broad-host-range vector, and derivatives thereof, for example, pAYC32 (Chistorerdov, A. Y., Tsygankov, Y. D. Plasmid, 16, 161-167 (1986)), pMFY42 (Gene, 44, 53 (1990)), pRP301, and pTB70 (Nature, 287, 396, (1980)). Examples thereof also include pBBR1 which is a broad-host-range vector having a different incompatibility from RSF1010, and derivatives thereof, such as pBHR1 (Antoine, R. and Loch, C., Molecular Microbiology, 6, 1785-99. (1992)).

[0039] To prepare a recombinant DNA via ligation of dapA*, lysC*, and DNAs encoding another protein into a vector that functions in bacteria belonging to the genus *Methylophilus*, the vector is digested with a restriction enzyme suitable for the terminus of a DNA fragment containing the genes. The ligation is usually performed with a ligase such as T4 DNA ligase. The genes may be introduced by separate vectors or the same vector.

The usual methods well known to those with skill in the art can be used as methods for digestion, ligation of DNA, preparation of chromosomal DNA, PCR, preparation of plasmid DNA, transformation, design of oligonucleotides used as primers, and the like. Such methods are described in Sambrook, J., Fritsch, E. F., and Maniatis, T., "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989) and the like.

[0040] Any method can be used to introduce a recombinant DNA prepared as described above into a bacterium belonging to the genus *Methylophilus*, so long as it provides sufficient transformation efficiency. For example, electroporation can be used (Canadian Journal of Microbiology, 43, 197 (1997)).

[0041] The DDPS and AK activities may also be enhanced by the presence of multiple copies of dapA* and lysC* on chromosomal DNA of a bacterium belonging to the genus *Methylobacillus*. Multiple copies of dapA* and lysC* may be introduced into the chromosomal DNA of a bacterium belonging to the genus *Methylobacillus* by homologous recombination, which can be performed by using a sequence present on chromosomal DNA in multiple copies as a target. A repetitive DNA or an inverted repeat present at the end of a transposable element can also be used as the sequence present on the chromosomal DNA in multiple copies. Alternatively, as disclosed in JP 02-109985 A, multiple copies of dapA* and/or lysC* can be introduced into the chromosomal DNA by incorporating them into a transposon and transferring it. In both of the methods, activities of DDPS and AK are amplified as a result of increased copy numbers of dapA* and lysC* in the transformant strains. In particular, the transposon is preferably a mini-Mu derived from Mu phage.

[0042] In addition to the above-described gene amplification methods, the DDPS activity and AK activity can be amplified by replacing expression control sequences such as promoters of *dapA** and *lysC**, with stronger ones (see JP 01-215280 A). Examples of known strong promoters include, for example, *lac* promoter, *trp* promoter, *trc* promoter, *tac* promoter, PR promoter of lambda phage, PL promoter, *tet* promoter, *amyE* promoter, and *spac* promoter. Use of these strong promoters enhances expression of *dapA** and *lysC**, and thus DDPS activity and AK activity are increased. Such enhancement of expression control sequences can be combined with increasing the copy number of *dapA** and *lysC**.

[0043] The bacterium of the present invention is not particularly limited so long as it is a methanol-assimilating bacterium which carries a DNA encoding a dihydrodipicolinate synthase which is desensitized to feedback inhibition by L-lysine and a DNA encoding a mutant LysE protein that promotes export of L-lysine to the outside of a bacterial cell when the DNA is introduced into a methanol-assimilating bacterium, wherein said bacterium is further modified to enhance intracellular activities of diaminopimelate dehydrogenase, diaminopimelate decarboxylase, dihydrodipicolinate reductase, and aspartate-semialdehyde dehydrogenase. Examples thereof include *Methylophilus methylotrophus* AJ110196 strain (FERM BP-10434) and microorganisms derived from AJ110196 (derivative strains). The term "derivative strains" as used herein refers to strains that are obtained from a parent strain AJ110196, which may be modified so that expression amounts of a DNA encoding a dihydrodipicolinate synthase which is desensitized to feedback inhibition by L-lysine and a DNA encoding a mutant LysE protein that promotes export of L-lysine to the outside of a bacterial cell when the DNA is introduced into a methanol-assimilating bacterium are further enhanced, or so that intracellular activities of diaminopimelate dehydrogenase, diaminopimelate decarboxylase, dihydrodipicolinate reductase, and aspartate-semialdehyde dehydrogenase are further enhanced, or as described below, may be modified so as to have another property.

[0044] In addition to the enhancement of expression of the above-mentioned six or seven kinds of genes, other enzymes involved in the L-lysine biosynthesis may also be enhanced. Such enzymes include diaminopimelic acid pathway enzymes (JP 2003-135066 A) such as phosphoenolpyruvate carboxylase (JP 60-87788 A), aspartate aminotransferase (JP 06-102028 B), and diaminopimelate epimerase; and an aminoadipic acid pathway enzyme such as homoaconitate hydratase. Moreover, activity of 6-phosphogluconate dehydratase or an Entner-Doudoroff pathway enzyme such as 2-keto-3-deoxy-6-phosphogluconate aldolase may be enhanced (JP 2004-166595 A).

[0045] Further, the methanol-assimilating bacterium of the present invention may have decreased activity of an enzyme that catalyzes a reaction that generates a compound other than L-lysine and branches off from the L-lysine biosynthetic pathway, or may be deficient in such an enzyme. Examples of the enzyme that catalyzes a reaction that generates a compound other than L-lysine and branches off from the L-lysine biosynthetic pathway include homoserine dehydrogenase (WO 95/23864) and L-lysine decarboxylase (JP 2004-254544 A). Modification to decrease enzymatic activities can be achieved by a technology such as gene disruption using homologous recombination.

The methanol-assimilating bacterium of the present invention may be further modified to be auxotrophic for L-methionine (JP 2004-248669 A). Such modification can be achieved by a natural mutation or a mutation treatment of the methanol-assimilating bacterium of the present invention so that the bacterium cannot grow in a medium containing no L-methionine or by disruption of *metA* gene (JP 2004-248669 A) or *metF* gene (SEQ ID NO: 58).

<3> Production of L-lysine

[0046] L-lysine can be produced by culturing the *Methylophilus* bacterium having L-lysine producing ability in a medium to produce and accumulate L-lysine in culture; and collecting L-lysine from the culture.

[0047] The microorganism used for the present invention can be cultured by a method typically used in culture of a methanol-assimilating microorganism. The medium used for the present invention may be either natural or synthetic so long as it contains a carbon source, nitrogen source, inorganic ions and other trace amount organic components as required.

[0048] If methanol is used as the main carbon source, L-lysine can be produced at a low cost. When methanol is used as the main carbon source, methanol is added to a medium in an amount of between 0.001 to 30%. As the nitrogen source, ammonium sulfate or the like is used by adding it to the medium. In addition to these, trace amount components such as potassium phosphate, sodium phosphate, magnesium sulfate, ferrous sulfate, and manganese sulfate can be added in small amounts.

[0049] The culture is performed under aerobic conditions by shaking, aeration by stirring, or the like at pH of between 5 to 9, and temperature of between 20 to 45°C, and the culture is typically complete within 24 to 120 hours. L-lysine can usually be collected from culture by a combination of ion exchange resin method, precipitation method, and other known methods.

[Examples]

[0050] The present invention will be further specifically explained hereinafter with reference to the following examples. The reagents used in the following examples were obtained from Wako Pure Chemicals Industries, Ltd. or Nacalai

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Tesque, Inc. unless otherwise indicated. The compositions of the medium used in each example are shown below. pH was adjusted with NaOH or HCl for all of the medium.

[0051]

5 (LB medium)
Tryptone/peptone (manufactured by Difco) 10 g/L
yeast extract (manufactured by Difco) 5 g/L
NaCl 10 g/L
pH 7.0
10 [These were steam-sterilized at 120°C for 20 minutes.]

[0052]

15 (LB agar medium)
LB medium
Bacto agar 15 g/L
[This was steam-sterilized at 120°C for 20 minutes.]

[0053]

20 (SEII medium)
K₂HPO₄ 1.9 g/L
NaH₂PO₄ 1.56 g/L
25 MgSO₄·7H₂O 0.2 g/L
(NH₄)₂SO₄ 5 g/L
CuSO₄·5H₂O 5 µg/L
MnSO₄·5H₂O 25 µg/L
ZnSO₄·7H₂O 23 µg/L
30 CaCl₂·2H₂O 72m g/L
FeCl₃·6H₂O 9.7m g/L
Methanol 0.5% (vol/vol) pH 7.0

35 [The components other than methanol were steam-sterilized at 121 °C for 15 minutes. After the components were sufficiently cooled, methanol was added.]

[0054]

40 (SEII production medium)
K₂HPO₄ 1.9 g/L
NaH₂PO₄ 1.56 g/L
MgSO₄·7H₂O 0.2 g/L
(NH₄)₂SO₄ 5 g/L
CuSO₄·5H₂O 5 µg/L
45 MnSO₄·5H₂O 25 µg/L
ZnSO₄·7H₂O 23 µg/L
CaCl₂·2H₂O 72 mg/L
FeCl₃·6H₂O 9.7 mg/L
Sodium pyruvate 2.5 g/L
50 CaCO₃ (manufactured by Kanto Chemical Co., Inc.) 30 g/L
Methanol 2% (vol/vol) pH 7.0

55 [The components other than methanol were steam-sterilized at 121°C for 15 minutes. After the components were sufficiently cooled, methanol was added.]

[0055]

(SEII agar medium)

	K ₂ HPO ₄	1.9 g/L
	NaH ₂ PO ₄	1.56 g/L
5	MgSO ₄ ·7H ₂ O	0.2 g/L
	(NH ₄) ₂ SO ₄	5 g/L
	CuSO ₄ ·5H ₂ O	5 µg/L
	MnSO ₄ ·5H ₂ O	25 µg/L
10	ZnSO ₄ ·7H ₂ O	23 µg/L
	CaCl ₂ ·2H ₂ O	72 mg/L
	FeCl ₃ ·6H ₂ O	9.7 mg/L
	Sodium pyruvate	1.0 g/L
15	Methanol	1% (vol/vol)
	pH 7.0	
	Bacto Agar (manufactured by Difco)	15 g/L

[The components other than methanol were steam-sterilized at 121 °C for 15 minutes. After the components were sufficiently cooled, methanol and if required, 20g/L L-methionine solution were added after filter sterilization.]

Example 1

<Construction of mini-Mu system, pMIV-Km, pMIV-Km-EA, pAET7>

[0056] A gene incorporation system using the bacteriophage Mu-phage isolated from *Escherichia coli* was used to increase the copy number of the lysE24 gene (a DNA encoding a mutant LysE protein) and the mutant dapA gene (a DNA encoding a dihydrodipicolinate synthase which is desensitized to feedback inhibition by L-lysine) on the chromosome.,.

<Construction of pMIV5> (Figs. 1 and 2)

[0057] In order to incorporate Mu-phage into the chromosome of *Escherichia coli*, an antibiotic resistance gene located between the recognition sequences (attL and attR) and a transferase required for transfer (Mu transposase) are required. It is not always necessary that both of them are incorporated into the same vector, and first, the pMIV5 plasmid was constructed, which contains the recognition sequences (attL and attR) and a kanamycin resistance gene, while the pAET7 plasmid with Mu transposase was constructed separately. Both of the plasmids can function when they are present in the same bacterium, resulting in the transfer of the region located between attL and attR to the chromosomal DNA of the strain. The pMIV5 plasmid was constructed as follows. First, the pMW119 vector (available from TOYOBO Co., Ltd.) is digested with the PvuII restriction enzyme and separated by agarose gel electrophoresis to collect a fragment of about 3.9 kbp. This fragment was ligated with DNA Ligation Kit (Takara Bio Inc.), to obtain the pMW1 plasmid. Subsequently, mini-Mu-phage was transferred to the pMW1 plasmid in *E. coli* cells. Specifically, the known plasmid pMD4041 (Journal of Bacteriology 158, 488-495 (1984)) was introduced into the *Escherichia coli* K12 strain, and a strain resistant to kanamycin and sensitive to ampicillin was selected to obtain a strain in which the plasmid pMD4041 was eliminated and mini-Mu 4041 was transferred to the chromosome. A factor that represses Mu transfer of mini-Mu4041, i.e., c repressor, has a temperature sensitive mutation. Therefore, when the strain is cultured at 42°C, c repressor of mini-Mu on the chromosome is deactivated, and transfer of mini-Mu4041 is significantly activated to cause effective transfer of mini-Mu to the chromosome, resulting in cell death. The strain with lysogenized mini-Mu4041 on the chromosome was transformed with pMW1 at 30°C. The strain with the plasmid pMW 1 was cultured in LB medium until the number of cells reached 2×10^8 cells/ml and treated at 42°C for 1 hour. In order to obtain a plasmid with mini-Mu4041 transferred to the pMW1 plasmid with this treatment, a plasmid DNA was prepared from the cells and used to transform an *Escherichia coli* strain. Plasmids were prepared from 50 strains of transformants having kanamycin resistance and ampicillin resistance, and the structures of the plasmids were determined by treating with a restriction enzyme, to thereby select the plasmid of interest. The plasmid was designated as pMu11. In the plasmid pMu11, mini-Mu4041 was transferred to the par region in the plasmid pMW1. More specifically, in pMW119 containing fragments of the known plasmids pBR322 and pSC101, mini-Mu4041 was inserted into the position 259, where the boundary position between the plasmids was defined as position 0. The plasmid was digested with the restriction enzyme HindIII and separated by agarose gel electrophoresis to collect a fragment of about 6.9 kbp, and the fragment was ligated with the DNA Ligation Kit (Takara Bio Inc.), to construct the pM12 plasmid (Fig. 1). The plasmid pM

12 was digested with HindIII-AvaII (EcoT22I) and used as a vector into which the terminator region of the PCR-amplified thr operon of *E. coli* was inserted. PCR amplification was performed using the chromosomal DNA of *E. coli* as the template and p-ter-thrL-f (SEQ ID NO: 1: 5-aaaaagcttaacacagaaaaagcc-3) and p-ter-thrL-r (SEQ ID NO: 2: 5-aaactgcagtggtc-gaaaaaaaagccc-3) as primers, under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 60 seconds. The resulting plasmid was digested with the restriction enzymes EcoRI-HindIII, and the PCR-amplified multi-cloning site region and the PCR-amplified p-factor independent transcription termination factor fragment from a bacteriophage fd were inserted into the plasmid. The multi-cloning site region was amplified by PCR using the plasmid pUC57 (Fermentus AB, available from LITHUANIA) as the template and using pUC57-MCS-f (SEQ ID NO: 3: 5-aaagaattcgagctcggtac-3) and pUC57-MCS-r (SEQ ID NO: 4: 5-aaaaagctt-gcatgcaggcctct-3) as primers, and PCR was performed under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 30 seconds. The amplified fragment was digested with EcoRI-BamHI, recognition sites of which had been added into the primer. The p-factor independent transcription termination factor fragment was amplified by PCR using a genomic DNA of the bacteriophage fd as a template and using ter-fd-f (SEQ ID NO: 5: 5-aaagatccgcatgccgtga-3) and ter-fd-r (SEQ ID NO: 6: 5-aaagaattccgatacaataaaggctccttttgagccttttttgagatttcaacgtgaaaaaattattattcgcaattccaagcta-3) as primers, and PCR was performed under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 30 seconds was repeated. The amplified fragment was digested with EcoRI-HindIII, recognition sites of which had been added into the primer. The three fragments were ligated with the DNA Ligation kit (Takada Bio Inc.) to construct the pMIV5 plasmid (Fig. 2). Then, the plasmid was digested with EcoRV, and a kanamycin resistance gene fragment obtained by cleaving a commercially-available plasmid (pUC4K) with HincII was inserted into the plasmid to construct the pMIV5-Km plasmid.

<pMIV-Km-lysE24dapA>

[0058] A gene of interest was inserted into the pMIV5-Km plasmid and used to incorporate the gene with mini-Mu, and the plasmid was used to transfer the gene fragment of interest to the chromosome. Specifically, the plasmid pMIV5-Km was digested with SmaI, followed by a dephosphorylation treatment. On the other hand, the fragment lysE24+dapA* was obtained by amplification through PCR using the known plasmid containing the pRSlysEdapA gene (JP 2003-61687 A), as a template and using pRS-1s (SEQ ID NO: 8: 5-cacagagacatattgccgttg-3) and dapA-r (SEQ ID NO: 7: 6-cattctagatccctaactttacagcaaacggcat-3) as primers. PCR was performed under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 120 seconds. PCR-amplified fragments were ligated with TaKaRa BKL kit (Takara Bio Inc.) to construct the pMIV-Km-lysE24dapA plasmid.

<Construction of pAET7> (Fig. 4)

[0059] The known plasmid pUC1918 (Gene, (1993) 134, 89-91) was digested with the restriction enzyme EcoRI, and the resulting fragment was blunt-ended. This fragment was used to insert the product obtained by a blunt-end treatment of a DNA fragment encoding Mu transposase, and a DNA fragment obtained by digesting the known plasmid pMu4041 (Journal of Bacteriology, (1984), 158, 488-495) with Scal-Eco47III. This plasmid was designated as pUC-MH7. The plasmid pUC-MH7 was digested with BamHI, and the resulting DNA fragment encoding Mu transposase was inserted into the BamHI site of the pAYC32 plasmid (Journal of General Microbiology 137, 169-178 (1991)) to obtain the plasmid pAET7.

Example 2

<Incorporation of the lysE24 and mutant dapA genes into the chromosome of *Methylophilus methylotrophus*, and the acquisition of VAE#1>

[0060] First, pAET7 was introduced into the *M. methylotrophus* AS strain by electroporation, and the bacterium was inoculated on an SEII plate containing 50 mg/l streptomycin. Then, the plasmid pMIV-Km-lysE24dapA was introduced into the resulting transformant to obtain strains that formed colonies on the SEII plate containing 20 mg/l kanamycin and 50 mg/l streptomycin. The mini-Mu cassette includes a kanamycin resistance gene, and the plasmid pMIV-Km-lysE24dapA cannot replicate in *M. methylotrophus*. Therefore, the kanamycin-resistant strain has the mini-Mu cassette inserted into the chromosome. Accordingly, 200 strains were selected randomly from these strains and spread onto an SEII plate containing 50 mg/l streptomycin and 20 mg/L kanamycin, followed by culturing at 37°C overnight. Then, bacterial cells present on the medium surface of about 0.3 square centimeters were scraped off and inoculated into an SEII production medium (5 ml) containing 50 mg/l streptomycin and 20 mg/L kanamycin, and the cells were cultured

with shaking at 37°C for 34 hours. After completion of the culture, the bacterial cells were removed by centrifugation, and the concentration of L-lysine in each culture supernatant was measured using Biotech-analyzer AS-210 (manufactured by Sakura Seiki Co., Ltd.). The strain containing the highest concentration of L-lysine was selected and designated as VAE#1.

Example 3

<Acquisition of a strain where lysE24 and mutant dapA genes are incorporated at higher copy numbers (VAE#8)>

[0061] The VAE#1 strain was considered to have one or two copies of a mini-Mu cassette inserted into the chromosome. Therefore, in order to improve productivity of L-lysine, the mini-Mu cassette was amplified on the chromosome. A gene encoding the MuC protein capable of suppressing the Mu transposase activity is on the plasmid pAET7 carrying the Mu transposase. The MuC protein is temperature sensitive, and therefore, when the strain is cultured at 42°C, the Mu transposase activity is promoted, resulting in amplification of the mini-Mu cassette on the chromosome. Specifically, the VAE#1 strain was suspended in an SEII liquid medium to an appropriate concentration, and the suspension was incubated at 42°C for 1 hour and diluted to appropriate concentrations. The bacterial solutions were inoculated onto SEII plates containing 50 mg/L streptomycin and 20 mg/L kanamycin to form single colonies. From the single colonies, 200 colonies were selected randomly and spread on SEII plates containing 50 mg/L streptomycin and 20 mg/L kanamycin, followed by culturing at 37°C overnight. Then, bacterial cells present on the medium surface of about 0.3 square centimeters were scraped off and inoculated in an SEII production medium (5 ml) containing 50 mg/L streptomycin and 20 mg/L kanamycin, and the cells were cultured with shaking at 37°C for 34 hours. After completion of the culture, the bacterial cells were removed by centrifugation, and the concentration of L-lysine in each culture supernatant was measured using Biotech-analyzer AS-210 (manufactured by Sakura Seiki Co., Ltd.) to select the strain with the highest concentration of L-lysine. The strain was designated as VAE#2. The procedure was repeated 8 times to obtain the VAE#8 strain. The amount of Lys produced by VAE#1 was defined as 100, and the relative value of the amount of Lys produced by VAE#8 strain was calculated and is shown in Table 1.

[0062]

[Table 1]

Name of strain	Relative amount of produced Lys (%)
VAE#1	100
VAE#8	800

Example 4

<Determination of transfer site in VAE#8>

[0063] Next, the site to which the mini-Mu cassette was transferred onto the chromosome of the VAE#8 strain was determined. The chromosomal DNA of the VAE#8 strain was prepared and completely digested with the restriction enzyme Sall. The resulting fragment was ligated to the pHSG398 vector, and selection was performed in an LB agar medium containing 12.5 mg/L chloramphenicol and 25 mg/L kanamycin to prepare a plasmid DNA from the resulting colonies. There are a kanamycin resistance gene on the mini-Mu cassette and chromosomal DNA around the transfer site in the plasmid. The nucleotide sequence of the plasmid was determined using a sequencing primer (attR-r1 5'-catctgttcattgaagcgcaagcta-3': SEQ ID NO: 9), which was designed outwardly in the inside of attR present in the right hand edge of the mini-Mu cassette, to thereby determine a transfer site of the mini-Mu cassette transferred to the chromosome. It is also possible to construct a strain identical to VAE#8 based on the information of the transfer region determined by the above-described method.

Example 5

<Imparting Met-auxotrophy to VAE#8 (#403)>

[0064] Next, methionine auxotrophy was imparted to the VAE#8 strain. Imparting amino acid auxotrophy to an amino acid-producing bacterium is effective to control the number of bacterial cells during the culture. The VAE#8 strain was subjected to an NTG-mutation treatment by the known method (WO 00/61723) and appropriately diluted to a cell density to form a single colony, and the bacterium was inoculated on an SEII agar medium containing 0.5 g/L L-methionine. The

cells were replicated on an SEII agar medium containing no L-methionine to obtain a strain that could not grow on the plate, that is, a strain auxotrophic for L-methionine. The strain was designated as #403. A plurality of genes from the #403 strain, which was considered to be involved in biosynthesis of L-methionine, were cloned by a method well-known by a person skilled in the art based on the homology to another microorganism to determine the nucleotide sequences. As a result, it was found that part of the *metF* gene encoding 5,10-methylenetetrahydrofolate reductase was deleted. Specifically, it was found that a region between the 92nd nucleotide and the 344th nucleotide, as counted from the initiation codon of the *metF* gene was deleted. It was possible to impart the L-methionine auxotrophy by disrupting the *metF* gene of VAE#8 by the known method disclosed by the inventors of the present invention (JP 2004-229662 A, Homologous recombination method using linear DNA). Details are described in Example 19. The strain with artificially disrupted *metF* was found to have the same properties as a strain auxotrophic for L-methionine obtained by the above-described NTG-mutation treatment, #403. A DNA fragment for gene disruption was prepared by Over-lap-PCR disclosed in the known document (Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R., *Gene*, 77, 51-9. (1989).). When the #403 strain and the control strain VAE#8 were cultured in an SEII production medium containing 0.075 g/L L-methionine and 2.5 g/L sodium pyruvate, the amount of accumulated Lys was increased. The amount of Lys produced by the VAE#8 strain was defined as 100, and the relative value of the amount of Lys produced by #403 strain was calculated and is shown in Table 2.

[0065]

[Table 2]

Name of strain	Relative amount of produced Lys (%)
VAE8	100
#403	156

Example 6

<Construction of plasmids pMIV-FRTGmFRT and pMIV-FRTGmFRT-EA> (Fig. 3)

[0066] In order to further incorporate a mini-Mu cassette into the #403 strain, an insertion cassette having a gene resistant to an antibiotic other than kanamycin was constructed. Specifically, the plasmid pMIV5 was digested with *EcoRV* and used as a vector. Then, the pKD4 plasmid (PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (2000) 97, 6640-6645) was digested with *HindIII-NdeI*, and the resulting fragment was blunt-ended, followed by insertion of a fragment having the kanamycin resistance gene region. The plasmid was designated as pMIV-FRTKmFRT. The plasmid was digested with the restriction enzyme *BglIII*, and the resulting fragment was blunt-ended, followed by insertion of the PCR-amplified gentamicin resistance gene fragment. PCR was performed using the pML122 plasmid (*Gene*, 89, 37-46. (1990)) as a template and using pGm-f (5-cgccagccaggacagaaatgc-3: SEQ ID NO: 10) and pGm-r (5-gtccagcggttttcttgggct-3: SEQ ID NO: 11) as primers, under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 60 seconds. The plasmid was designated as pMIV-FRTGmFRT. A gene of interest was inserted into the region between attL and attR to construct a mini-Mu cassette to amplify the gene of interest on a chromosome of *M. methylotrophus* (Fig. 3). Specifically, the plasmid pMIV-FRTGmFRT was digested with the restriction enzyme *SmaI*, followed by a dephosphorylation treatment. On the other hand, the *lysE24+dapA** fragment was obtained by amplification with PCR using the known plasmid comprising the pRSlysEdapA gene (JP 2003-61687 A) as a template and using pRS-1s and *dapA-r* as primers. PCR was performed under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 120 seconds. PCR-amplified fragments were phosphorylated using TaKaRa BKL kit (Takara Bio Inc.) and ligated to a vector, to thereby construct the plasmid pMIV-FRTGmFRT-EA. The plasmids pKD4 and pCP20 were registered at the E.coli Genetic Stock Center as CGSC strain #7632 and #7629, respectively, and it is available from the Center.

Example 7

<Acquisition of strain in which the *lysE24* and mutant *dapA* genes are incorporated at higher copy numbers using pMIV-Gm-EA (#403-11-Gm)>

[0067] First, pAET7 was introduced into the #403 strain, and the cells were inoculated onto an SEII plate containing 50 mg/l streptomycin. Then, the pMIV-FRTGmFRT-EA plasmid was introduced into the resulting pAET7 transformant by electroporation to obtain strains that formed colonies on an SEII plate containing 50 mg/l streptomycin. The mini-Mu

cassette includes a gentamicin resistance gene, and the plasmid pMIV-FRTGmFRT-EA cannot replicate in *M. methylotrophus*. Therefore, the gentamicin-resistant strain has the mini-Mu cassette inserted into the chromosome. Accordingly, 200 strains were selected randomly from these strains and spread on an SEII plate containing 50 mg/L streptomycin and 20 mg/L gentamicin, followed by culturing at 37°C overnight. Then, bacterial cells present on the medium surface of about 0.3 square centimeters were scraped off and inoculated in an SEII production medium (5 ml) containing 50 mg/L streptomycin and 20 mg/L gentamicin, and the cells were cultured with shaking at 37°C for 48 hours. After completion of the culture, the bacterial cells were removed by centrifugation, and the concentration of L-lysine in each culture supernatant was measured using Biotech-analyzer AS-21 0 (manufactured by Sakura Seiki Co., Ltd.) to select the strain with the highest concentration of L-lysine. The strain was designated as #403-11Gm.

Example 8

<Determination of transfer site in #403-11-Gm>

[0068] Next, the site to which the mini-Mu cassette was transferred on the chromosome of the #403-11-Gm strain was determined. A chromosomal DNA of #403-11 strain was prepared and completely digested with the restriction enzyme Sall. The resulting fragment was ligated to the vector pHSG398, and selection was performed in an LB agar medium containing 12.5 mg/L chloramphenicol and 25 mg/L gentamicin to prepare a plasmid DNA from the resulting colonies. There are a cloned kanamycin resistance gene on a mini-Mu cassette and chromosomal DNA around the transfer site in the plasmid. The nucleotide sequence of the plasmid was determined using a sequencing primer (attR-r1 5'-catctgttcatttgaagcgcgaagcta-3'; SEQ ID NO: 12), which was designed in the attR region present in the right hand edge of the mini-Mu cassette, to thereby determine the transfer site of the mini-Mu cassette which had been transferred to the chromosome. It is also possible to construct a strain identical to #403-11 Gm based on the information of the transfer region determined by the above-described method.

Example 9

<Elimination of the antibiotic-resistant marker from #403-11-Gm (pFLP31), and acquisition of #403-11 strain>

<Construction of pAYCTER3>

[0069] Synthetic DNAs of SEQ ID NO: 13 (5'-aattcgagct cggtagccgg ggatcctcta gagtcgacct gcagggatgc aagctta-3') and SEQ ID NO: 14 (5'-gatctaagct tgcattgctg caggtcgact ctaggagatc cccgggtacc gagctcg-3'), designed so as to contain a sequence of a multi-cloning site of pUC19, were annealed by well-known method to produce a polylinker. The polylinker was designed so as to have the same terminal as that of the fragment cleaved with restriction enzymes EcoRI and BglII. Moreover, primers of SEQ ID NO: 15 (5'-ctatgatcat ttgctgtgcg gcagtagcgc-3') and SEQ ID NO: 16 (5'-cttagatctcaaaa-gagttgtagaacgc-3') were synthesized, and the region encoding the *rrnB* terminator sequence was amplified by PCR from chromosomal DNA of *Escherichia coli* K-12 strain prepared by a conventional method (method of Saito and Miura [Biochim. Biophys. Acta, 72, 619 (1963)]). The primers of SEQ ID NO: 13 and SEQ ID NO: 14 were designed so as to have the recognition sequence of the restriction enzyme BglII and a recognition sequence of a restriction enzyme *BclI*, respectively. PCR was performed using Pyrobest DNA polymerase (manufactured by Takara Bio Inc.) under reaction conditions recommended by the manufacturer. The resulting PCR fragment was digested with restriction enzymes BglII and *BclI*, and the PCR fragment was ligated to the above-mentioned polylinker, to thereby produce a DNA fragment of about 400 bp. The ligation reaction was performed with the DNA Ligation Kit Ver.2.1 (manufactured by Takara Bio Inc.) under reaction conditions recommended by the manufacturer. Then, a fragment of about 9.2 kbp, obtained by cleaving the pAYC32 plasmid (J. Gen. Microbiol., 137, 169-178 (1991)) with restriction enzymes EcoRI and BamHI, was collected, and the above-mentioned DNA fragment was inserted therein to construct an expression plasmid pAYCTER3 capable of functioning in *M. methylotrophus* NCIMB 10515. The pAYCTER3 lacks a sequence upstream on the 5' side of *strA* gene encoded in pAYC32 and alternatively has the pUC 19 multi-cloning site and the *rrnB* terminator.

<Construction of pFLP31>

[0070] The gentamicin resistance gene of the #403-11Gm strain constructed in Example 7 was designed so that it is located between two FRT sequences, so the antibiotic resistance gene can be eliminated from the chromosome by a reaction with an FLP recombinase. The pAYCTER3 constructed by the above-mentioned method was digested with BamHI-SmaI, and a 3.3-kbp fragment obtained by cleaving the pCP20 plasmid (PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (2000) 97, 6640-6645) with SmaI-BamHI containing an FLP recombinase was inserted therein. The resulting plasmid was designated as pFLP31. The plasmid

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pCP20 was registered at the E.coli Genetic Stock Center as CGSC strain #7629, and it is available from the Center.

<Elimination of antibiotic-resistance marker>

[0071] The pAET7 was eliminated from #403-11Gm by a known method to obtain a streptomycin-sensitive strain. The above-mentioned plasmid pFLP31 was introduced into the strain by electroporation, and the cells were inoculated on an SEII agar medium containing 50 mg/L streptomycin and 0.5 g/L L-methionine. The resulting strain was suspended to an appropriate concentration in an SEII agar medium containing 50 mg/L streptomycin and 0.5 g/L L-methionine. The suspension was heated at 42°C for 1 hour and diluted to form single colonies, and the cells were inoculated on an SEII agar medium containing 50 mg/L streptomycin and 0.5 g/L L-methionine. From the colonies, strains sensitive to gentamicin were selected. Then, pAET7 was eliminated from the strain to obtain a streptomycin-sensitive strain, which was designated as #403-11. When the #403-11 strain and a control strain (#403 strain) were cultured in an SEII production medium containing 0.075 g/L L-methionine and 2.5 g/L sodium pyruvate, the amount of accumulated Lys was increased. The amount of Lys produced by #403 strain was defined as 100, and the relative value of the amount of Lys produced by #403-11 strain was calculated and shown in Table 3.

[0072]

[Table 3]

Name of strain	Relative amount of produced Lys (%)
#403	100
#403-11	108

Example 10

<Confirmation of increased DDPS activity>

[0073] In the #403-11 obtained as described above, 8 copies of a lysE24dapA cassette has been transferred to the chromosome. Therefore, an increase in the activity of a dihydrodipicolinate synthase encoded by dapA was confirmed for #403-11 strain. The dihydrodipicolinate synthase activity was measured in accordance with a modified method of the known method (Journal of Biological Chemistry, 240, 4710-4716 (1965)). Specifically, a reaction solution was prepared so as to contain 50 mM imidazole-HCl (pH 7.4), 2 mM L-aspartate-β-semialdehyde, 2 mM sodium pyruvate, and a cell extract, and the final volume of the solution was adjusted to 1 ml. The results are shown in Table 4.

[0074]

[Table 4]

Name of bacterial strain	Specific activity (milliunit/milligram protein)
AS1	12
#403-11	129

The amount of an enzyme that produces 1 micromol of a product per minute was defined as 1 unit.

Example 11

<Construction of plasmid pBGEA, and construction of #403-11/pBGEA>

(1) Construction of plasmid pBGEA carrying L-lysine biosynthetic enzyme gene (dapA*) and a gene having L-lysine export activity (lysE24)

[0075] In order to introduce the dapA* and LysE24 genes into a bacterium belonging to the genus *Methylophilus*, the pBHR1 plasmid (Antoine, R. and Loch, C., Molecular Microbiology, 6, 1785-99. (1992)) was used to construct the pBGEA plasmid for expressing dapA* and LysE24. First, pBHR1 was digested with a restriction enzyme DraI, and the resulting fragment was added to a phenol/chloroform solution and mixed to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected, and DNA was collected by ethanol precipitation. The resulting DNA fragment was blunt-ended using DNA Blunting kit (manufactured by Takara Shuzo).

On the other hand, dapA* and LysE24 genes were obtained from the pRSlysEdapA plasmid containing the genes (JP

2003-61687 A). *E. coli* JM109 strain transformed with the plasmid pRSlysEdapA was designated as AJ13832 and deposited at National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository on June 4, 2001 under the accession number of FERM P-18371. Then, the deposit was converted to an international deposition under the provisions of the Budapest Treaty on May 13, 2002 under the accession number of FERM BP-8042. First, pRSlysEdapA was digested with restriction enzymes EcoRI and BglII, and the resulting fragment was added to a phenol/chloroform solution and mixed to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected, and DNA was collected by ethanol precipitation. Then, the DNA fragment of interest was separated by electrophoresis on 0.8% agarose gel, and the DNA fragment of about 2.0 Kbp was collected by using EASY TRAP ver. 2 (DNA collection kit, manufactured by Takara Shuzo). The resulting DNA fragment was blunt-ended by using BKL kit (manufactured by Takara Shuzo) and phosphorylated.

The digestion product of pBHR1 and the dapA* and LysE24 gene region fragments prepared as described above were ligated with the DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo). This ligation product was used to transform *Escherichia coli* (*E. coli* JM109 competent cells, manufactured by Takara Shuzo), and the cells were inoculated on an LB agar medium containing 20 mg/L of kanamycin and incubated overnight at 37°C. The colonies that appeared on the agar medium were inoculated in an LB liquid medium containing 20 mg/L of kanamycin and cultured at 37°C for 8 hours with shaking. Plasmid DNA was extracted from each culture medium by the alkali-SDS method, and structure of each plasmid was confirmed by digestion with restriction enzymes and determination of the nucleotide sequence. A plasmid with identical transcription directions of the chloramphenicol resistance gene and the dapA* and lysE24 genes was selected as pBHR-EA.

A gentamicin resistant marker was introduced into pBHR-EA obtained as described above to construct the pBGEA plasmid. First, pBHR-EA was digested with a restriction enzyme NcoI, and the resulting fragment was added to a phenol/chloroform solution and mixed to terminate the reaction. After the reaction mixture was centrifuged, the upper layer was collected, and DNA was collected by ethanol precipitation. The resulting DNA fragment was blunt-ended by using DNA Blunting kit (manufactured by Takara Shuzo). On the other hand, the gentamicin resistance gene region was amplified by PCR using the pML122 plasmid (Monika Labes, Alfred Puhler, and Reinhard Simon, Gene, 89, (1990), 37-46) as a template DNA and using pGm-f (SEQ ID NO: 17: 5-CGCCAGCCAGGACAGAAATGC-3) and pGm-r (SEQ ID NO: 18: 5-GTCCAGCGTTTTTCTTGGGCT-3) as primers, and PCR was performed under the following conditions: denaturation at 94°C for 10 seconds, annealing at 60°C for 30 seconds, and extension reaction at 72°C for 90 seconds. The PCR was performed using Pyrobest DNA polymerase (manufactured by Takara Shuzo). The resulting gentamicin resistance gene fragment was blunt-ended by using BKL kit (manufactured by Takara Shuzo) and phosphorylated.

The digestion product of pBHR-EA and the gentamicin resistance gene region fragment prepared as described above were ligated by using DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo). This ligation product was used to transform *Escherichia coli* (*E. coli* JM109 competent cells, manufactured by Takara Shuzo), and the cells were inoculated on an LB agar medium containing 20 mg/L of gentamicin and incubated overnight at 37°C. The colonies that appeared on the agar medium were inoculated in an LB liquid medium containing 20 mg/L of gentamicin and cultured at 37°C for 8 hours with shaking. Plasmid DNA was extracted from each culture medium by the alkali-SDS method, and structure of each plasmid was confirmed by digestion with restriction enzymes and determination of the nucleotide sequence to obtain pBGEA. The pBGEA was further introduced into #403-11 strain prepared in Example 9, in which the lysE24 and dapA* genes had been incorporated into the chromosome, to enhance lysE24 and dapA* genes. The strain was designated as #403-11/pBGEA. When the #403-11/pBGEA and a control strain #403-11 were cultured in an SEII production medium containing 20 mg/L kanamycin, 50 mg/L gentamicin (the medium for the control strain contained no gentamicin), 0.075 g/L L-methionine, and 2.5 g/L sodium pyruvate, the amount of produced Lys was increased. The amount of Lys produced by #403-11 strain was defined as 100, and the relative value of the amount of Lys produced by #403-11/pBGEA strain was calculated and shown in Table 5. It was found that introduction of the plasmid increased the amount of Lys produced by #403-11 strain.

[0076]

[Table 5]

Name of strain	Relative amount of produced Lys (%)
#403-11	100
#403-11/pBGEA	111

Example 12

<Construction of plasmids pRSlysA, pRSddh, pRSdapB, pRSasd, and pRSask, introduction of the plasmids into

#403-11/pBGEA strain, and evaluation of L-lysine productivity>

[0077] Next, expression plasmids carrying each Lys biosynthetic gene were constructed, and the plasmids were introduced into #403-11/pBGEA strain, and the effects on L-lysine productivity were investigated.

<1> Construction of plasmid pRSlysA

[0078] The diaminopimelate decarboxylase gene (*lysA*) from *Methylophilus methylotrophus* was obtained by PCR using two oligonucleotide primers prepared based on the known sequence (SEQ ID NO: 13 in WO2000/061723) and using a chromosomal DNA of *Methylophilus methylotrophus* as a template. PCR was performed using *plysA-f* (SEQ ID NO: 19: 5'-AAACCCGGGGATCCTGAGCGCCAATACCCTCAAACGCCT-3') and *plysA-r* (SEQ ID NO: 20: 5'-TTTCCCGGGCTTGCGGCTTCGGTTTTTTATTAGGGTTGCC-3') as primers, under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 60 seconds. The amplified *lysA* gene fragment from *M. methylotrophus* was digested with *Sse8387I-XbaI*, recognition sites of which had been added into the primer sequence, and the resulting fragment was ligated to the pRStac plasmid (JP 2003-61687 A) which had been digested with *Sse8387I-XbaI*. The plasmid was designated as pRSlysA.

<2> pRSddh

[0079] The diaminopimelate hydrogenase gene (*ddh*) from *Brevibacterium lactofermentum* was obtained by amplification with PCR using two oligonucleotide primers prepared based on the known nucleotide sequence of *ddh* of *Corynebacterium glutamicum* (Ishino, S. et al. Nucleic acid res. 15, 3917 (1987)) and using a chromosomal DNA of *Brevibacterium lactofermentum* 2256 strain (ATCC 13869 strain) as a template. PCR was performed using *pddh-f* (SEQ ID NO: 21: 5'-ACCCCTGCAGGGCCACCACAATTTTGGAGGATTACAAGAAC-3') and *pddh-r* (SEQ ID NO: 22: 5'-TC-CTCTAGACTCGAGCTAAATTAGACGTCGCGT-3') as primers, under the following conditions: a cycle consisting of denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 60 seconds was repeated for 25 cycles. The amplified *ddh* gene fragment was digested with *Sse8387I-XbaI*, recognition sites of which had been added into the primer sequence, and the resulting fragment was ligated to the pRStac plasmid (JP 2003-61687 A) which had been digested with *Sse8387I-XbaI*. The plasmid was designated as pRSddh.

<3> pRSdapB

[0080] The dihydrodipicolinate reductase gene (*dapB*) from *E. coli* was amplified by PCR using two oligonucleotide primers prepared based on the known nucleotide sequence and using a chromosomal DNA of *E. coli* as a template. PCR was performed using *pdapB-f* (SEQ ID NO: 23: 5'-GCGCCTGCAGGCGCTGGTTACTCTGAAAACGGTCT-3') and *pdapB-r* (SEQ ID NO: 24: 5'-GCATCTAGAGACAATTTAAAAACATAACACCAAAAATAAAAGGGCC-3') as primers, under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 60 seconds. The amplified *dapB* gene fragment was digested with *Sse8387I-XbaI*, recognition sites of which had been added into the primer sequence, and the resulting fragment was ligated to the pRStac plasmid (JP 2003-61687 A) which had been digested with *Sse8387I-XbaI*. The plasmid was designated as pRSdapB.

<4> pRSlasd

[0081] The aspartate-semialdehyde dehydrogenase gene (*asd*) from *E. coli* was amplified by PCR using two oligonucleotide primers prepared based on the known nucleotide sequence using a chromosomal DNA of *E. coli* as a template. PCR was performed using *pasd-f* (SEQ ID NO: 25: 5'-GCCCCTGCAGGCCGGCACATTTATACAGCACATCTTTG-3') and *pasd-r* (SEQ ID NO: 26: 5'-TAATCTAGAAAGATTACGCCAGTTGACGAAGCATC-3') as primers, under the following conditions: a cycle consisting of denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 60 seconds was repeated for 25 cycles. The amplified *ddh* gene fragment was digested with *Sse8387I-XbaI*, recognition sites of which had been added into the primer sequence, and the resulting fragment was ligated to the pRStac plasmid (JP 2003-61687 A) which had been digested with *Sse8387I-XbaI*. The plasmid was designated as pRSasd.

<5> pRSask

[0082] The aspartokinase gene (*ask*) from *Methylophilus methylotrophus* was obtained by PCR using two oligonucleotide primers prepared based on the known sequence (SEQ ID NO: 5 in WO2000/061723) and using a chromosomal DNA of *Methylophilus methylotrophus* as a template. PCR was performed using *pask-f* (SEQ ID NO: 27: 5'-

AGGGAATTCTAAACCGGATATGGCGATGGCAGGTGGTACT-3') and pask-r (SEQ ID NO: 28: 5'-TAACTGCAG-GAAGTTTAAATAGTACCAACACAGCGCATG-3') as primers, under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 90 seconds. The amplified ask gene fragment from *M.methylotrophus* was digested with Sse8387I-XbaI, recognition sites of which had been added in primer and blunt-ended, and the resulting fragment was ligated to the pRStac plasmid (JP 2003-61687 A) which had been digested with Sse8387I and blunt-ended. The plasmid was designated as pRSask.

<6> Introduction and evaluation of the plasmids

[0083] The five plasmids prepared as described above were separately introduced into #403-11/pBGEA strain prepared in Example 11, and the resulting strains were cultured in an SEII production medium containing 0.075 g/L L-methionine and 2.5 g/L sodium pyruvate. However, Lys productivity was not improved in all the strains. In the strain with ddh, Lys productivity was reduced.

Example 13

<Enhancement of ddh+lysA in combination, construction of plasmid pDA, and evaluation of productivity of L-lysine>

[0084] In order to determine the next limiting factor in Lys production using #403-11 strain which was modified so that expression of lysE24 and dapA* genes effective for improving Lys production in *M.methylotrophus* was sufficiently enhanced by gene incorporation and plasmid introduction, the genes were separately enhanced as shown in Example 12 to investigate the effects, but the inventors of the present invention could not find an effective gene. Therefore, the inventors constructed various plasmids for enhancement of the genes in combination, each carrying two genes, and introduced into #403-11 strain to evaluate the resulting strains. As a result, the inventors found that enhancing lysA and ddh in combination improved productivity of L-lysine.

It was thought that diaminopimelate dehydrogenase encoded by ddh reversibly catalyzes both of production reaction and degradation reaction of diaminopimelic acid, and that enhancement of ddh alone promoted not only production but also degradation of diaminopimelic acid. On the other hand, diaminopimelate decarboxylase which catalyzes the reaction subsequent to that of the diaminopimelate dehydrogenase is an irreversible enzyme that causes decarboxylation reaction forward L-lysine production. It was thought that enhancement of both diaminopimelate dehydrogenase and diaminopimelate decarboxylase prevented degradation of a diaminopimelic acid by diaminopimelate dehydrogenase and promoted synthesis of the diaminopimelic acid.

Specifically, the plasmid pRSddh was digested with SapI, and the resulting fragment was blunt-ended and dephosphorylated to produce a vector, into which a PCR-amplified DNA fragment including the lysA gene region containing the tac promoter region was inserted. PCR amplification was performed using the pRSlysA plasmid as a template and using ptac-f (SEQ ID NO: 29: 5'-AAAAGATCTCCCGTTCTGGATAATGTTTTTTCGCGCCGAC-3') and plysA-r (SEQ ID NO: 20: 5'-TTTCCCGGGCTTGGCGGCTTCGGTTTTTTTATTAGGGGTTGCC-3') as primers, under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 60 seconds. The resulting PCR-amplified fragment was digested with XbaI, and the resulting fragment was blunt-ended and ligated to the above-mentioned vector. In the resulting plasmid, the transcription directions of the ddh and lysA genes were identical to each other, and the plasmid was designated as pDA. When the strain obtained by introducing the plasmid pDA into #403-11/pBGEA strain (#403-11/pBGEA/pDA) and the control strain #403-11/pBGEA were cultured in an SEII production medium containing 20 mg/L kanamycin, 50 mg/L gentamicin, and 50 mg/L streptomycin (the medium for the control strain contained no streptomycin), 0.075 g/L L-methionine, and 2.5 g/L sodium pyruvate, the amount of produced Lys was increased. The amount of Lys produced by #403-11/pBGEA strain was defined as 100, and the relative value of the amount of Lys produced by #403-11/pBGEA/pDA strain was calculated and shown in Table 6.

[0085]

[Table 6]

Name of strain	Relative amount of produced Lys (%)
#403-11/pBGEA	100
#403-11/pBGEA/pDA	120

Example 14

<Enhancement of further L-lysine biosynthetic genes in combination, construction of pBDAS (lysA+ddh+dapB+asd),

and evaluation of L-lysine productivity>

[0086] It was found that the L-lysine productivity was improved by enhancing enzyme genes, *ddh* and *lysA*, which are capable of catalyzing two sequential reactions in the biosynthesis pathway, in combination. The inventors of the present invention investigated the effects of enhancement of other enzyme genes in combination with *ddh*+*lysA* and found that the use of *dapB* and *asd* in combination with *ddh* and *lysA* improved L-lysine production.

Specifically, the pRS*dapB* plasmid was digested with *EcoRI*, and the resulting fragment was blunt-ended and dephosphorylated to prepare a vector. The plasmid pDA was digested with *HpaI*-*SapI*, and a resulting DNA fragment of 2.5 kbp including *lysA* and *ddh* each having a *tac* promoter upstream of the genes was collected and blunt-ended to be ligated to the vector, to thereby construct the plasmid pBDA. The plasmid was found to have *ddh* and *lysA* genes inserted upstream of *dapB* so that the direction of *ddh* and *lysA* and the direction of *dapB* were identical to each other. The plasmid pBDA was further digested with *SapI*, and the resulting fragment was blunt-ended and dephosphorylated to prepare a vector, into which a PCR-amplified DNA fragment including the *asd* gene region containing the *tac* promoter region was inserted. PCR amplification was performed using the plasmid pRS*asd* as a template and using *ptac-f* and *pasd-r* as primers, under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 90 seconds. The resulting PCR-amplified fragment was blunt-ended and phosphorylated, and the fragment was ligated to the above-mentioned vector. The resulting plasmid was designated as pBDAS. When a strain obtained by introducing the plasmid pBDAS into #403-11/pBGEA strain (#403-11/pBGEA/pBDAS) and a control strain #403-11/pBGEA were cultured in an SEII production medium containing 20 mg/L kanamycin, 50 mg/L gentamicin, and 50 mg/L streptomycin (the medium for the control strain contained no streptomycin), 0.075 g/L L-methionine, and 2.5 g/L sodium pyruvate, the amount of produced Lys was increased. The amount of Lys produced by #403-11/pBGEA strain was defined as 100, and the relative value of the amount of Lys produced by #403-11/pBGEA/pBDAS strain was calculated and shown in Table 7.

[0087]

[Table 7]

Name of strain	Relative amount of produced Lys (%)
#403-11/pBGEA	100
#403-11/pBGEA/pDA	120
#403-11/pBGEA/pBDAS	143

Example 15

<Construction of pCBDAS (*lysA*+*ddh*+*dapB*+*asd*+*lysC*), introduction of the plasmid into #403-11/pBGEA strain, and evaluation of L-lysine productivity>

[0088] There was not a restriction site suitable for introduction of an additional biosynthetic gene in the plasmid pBDAS, and it was difficult to insert an additional gene, and therefore, the inventors solved the problem by cloning genes of interest in a vector pMW 119 and cleaving the genes as a whole. First, the vector pMW119 was digested with *XbaI*, and the resulting fragment was ligated to a DNA fragment comprising *Ptac*+*dapB* which had been obtained by cleaving pRS*dapB* with *XbaI*. Subsequently, the plasmid was digested with *BamHI*, and the resulting fragment was blunt-ended and dephosphorylated to prepare a vector, which was then ligated to a DNA fragment comprising *Ptac*+*ddh*+*Ptac*+*lysA* obtained by cleaving the pDA plasmid with *HpaI*-*SapI*. The plasmid was digested with *SmaI*, and the resulting fragment was dephosphorylated to prepare a vector, which was then ligated to a DNA fragment including *Ptac*+*asd* prepared in the same way as Example 14. The plasmid constructed as described above was designated as pMWBDAS. In the plasmid, the directions of *ddh*, *lysA*, and *asd* were identical to each other, while only the direction of *dapB* was the opposite. The *dapB*, *ddh*, *lysA*, and *asd* genes are located on pMW119 in the stated order from the direction of the *lac* promoter. When the plasmid is digested with *Sall*-*SacI*, a fragment containing *dapB*, *ddh*, *lysA*, and *asd* each containing a *tac* promoter is obtained. The above-mentioned plasmid pRS*ask* was digested with *SapI* and blunt-ended, and the fragment was ligated thereto, to thereby construct pCBDAS. When a strain obtained by introducing the plasmid pCBDAS into #403-11/pBGEA strain (#403-11/pBGEA/pCBDAS) was cultured in an SEII production medium containing 20 mg/L kanamycin, 50 mg/L gentamicin, 50mg/L streptomycin (the medium for the control strain contained no streptomycin), 0.075 g/L L-methionine, and 2.5 g/L sodium pyruvate and supplemented with ammonium sulfate to a final concentration of 6 g/L, the amount of produced Lys was increased. The amount of Lys produced by #403-11/pBGEA strain was defined as 100, and the relative value of the amount of Lys produced by #403-11/pBGEA/pBDAS strain was calculated and shown in Table 8.

[0089]

[Table 8]

Name of strain	Relative amount of produced Lys (%)
#403-11/pBGEA	100
#403-11/pBGEA/pDA	120
#403-11/pBGEA/pBDAS	143
#403-11/pBGEA/pCBDAS	160

Example 16

<Construction of transfer unit for reconstruction of a producing bacterium capable of eliminating antibiotic-resistant marker (V12S α) and construction of V12S α strain (lysE24+dapA* gene-amplified strain)>

[0090] Next, a method of producing a strain containing no antibiotic-resistant gene marker and carrying Lys biosynthetic genes in the chromosome will be described. The plasmid pMIV-FRTKmFRT shown in Example 6 was digested with SmaI, and a PCR-amplified lysE+dapA* fragment was inserted therein. The lysE+dapA* fragment was obtained by the method shown in Example 6. The resulting plasmid was designated as pMIV-FRTKmFRT-EA. The plasmid has an antibiotic-resistant gene different from that of pMIV-FRTGmFRT-EA.

First, the plasmid pAET7 described in Example 1 was introduced into a wild-type strain of *M.methylotrophus* AS1 (NCIMB10515), and a transformant was selected in an SEII agar medium containing 50 mg/L streptomycin. The plasmid pMIV-FRTKmFRT-EA was introduced into the strain by electroporation in accordance with the method described in Example 2, and strains to which the mini-Mu cassette (EA unit) comprising lysE24+dapA* genes was transferred to chromosome were selected in an SEII agar medium containing 50 mg/L streptomycin and 20 mg/L kanamycin. From the strains, 200 strains were selected randomly and inoculated on SEII plates containing 50 mg/L streptomycin and 20 mg/L kanamycin, followed by culture at 37°C overnight. Then, bacterial cells present on the medium surface of about 0.3 square centimeters were scraped off and inoculated in an SEII production medium (5 ml) containing 50 mg/l streptomycin and 20 mg/L kanamycin, and the cells were cultured with shaking at 37°C for 34 hours. After completion of culture, the bacterial cells were removed by centrifugation, and the concentration of L-lysine in each culture supernatant was measured using Biotech-analyzer AS-21 0 (manufactured by Sakura Seiki Co., Ltd.) to select a strain with the highest concentration of L-lysine. The strain was designated as V1. Transfer was repeated 12 times in the same way as Example 3 to obtain V 12 strain. The amount of Lys produced by V 1 was defined as 100, and the relative value of the amount of Lys produced by V12 strain was calculated and shown in Table 9.

[0091]

[Table 9]

Name of strain	Relative amount of produced Lys (%)
V1	100
V12	1066

[0092] Next, the transfer site of the mini-Mu cassette on the chromosome of V12 strain was determined in accordance with completely the same method as in Example 4. Based on the information, it is possible to construct a strain completely identical to the V12 strain.

[0093] Subsequently, the plasmid pFLP31 shown in Example 9 was introduced into V 12 strain, and a kanamycin-resistant gene region was eliminated from the mini-Mu cassette on the chromosome by the method described in Example 9. The kanamycin-sensitive strain obtained by the above-mentioned procedure was designated as V12S α . The V12S α was designated as AJ 110196, and the strain was deposited as an international deposition under the provisions of Budapest Treaty at the National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary (Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-5466, Japan) on October 12, 2005 and received the accession number FERM BP-10434.

Example 17

<Construction of lysE24+dapA*+lysA+ddh+dapB+asd+lysC transfer unit>

[0094] As shown in Example 15, it was found that enhancement of various Lys biosynthetic genes in addition to enhancement of lysE24+dapA* improved Lys productivity, so a plasmid for incorporating these genes into the chromosome was constructed. First, pMIV-FRTKmFRT was digested with EcoRI, and the resulting fragment was blunt-ended and dephosphorylated, followed by insertion of a PCR-amplified lysE24+dapA* fragment therein to construct pMIV-FRTKmFRT-EA#1. The plasmid was digested with SmaI, and the resulting fragment was dephosphorylated to prepare a vector, followed by insertion of a PCR-amplified Ptac+dapB fragment. PCR amplification was performed using the pRSdapB plasmid as a template and using ptac-f and pdapB-r as primers, under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 90 seconds. The PCR-amplified fragment was blunt-ended and phosphorylated to be ligated to the above-mentioned vector to obtain pMIV-FRTKmFRT-EAB#1. The transcription directions of the dapA and dapB genes were identical to each other. Then, the plasmid was digested with HindIII, and the resulting fragment was blunt-ended and dephosphorylated, which was then ligated to a DNA fragment comprising lysA and ddh obtained by the method described in Example 14 to thereby obtain pMIV-FRTKmFRT-EABDA#12. The transcription direction of the ddh gene was opposite to that of dapA and dapB. The plasmid was digested with Sall, and the resulting fragment was blunt-ended and dephosphorylated, followed by insertion of a DNA fragment comprising an asd gene region containing a tac promoter region which had been prepared by the method described in Example 14. In this procedure, the asd fragment was inserted into the blunt-ended fragment, and therefore, a plasmid where two asd fragments were inserted was obtained and designated as pMIV-FRTKmFRT-EABDAS#10. The directions of both the asd genes were opposite to that of dapA and dapB. In the resulting pMIV-FRTKmFRT-EABDAS#10, the genes lysE, dapA, dapB, asd, asd, lysA, and ddh were located in the stated order. As described above, a plasmid for transferring a gene cassette containing six kinds of genes to chromosome was constructed.

Example 18

<Construction of lysE24+dapA*+lysA+ddh+dapB+asd+lysC-amplified strains (Vmac3 strain, Vmac3S strain) and evaluation of L-lysine productivity>

[0095] pAET7 was introduced into V12S α obtained in Example 16, and the pMIV-FRTKmFRT-EABDAS#10 plasmid was also introduced to obtain V12S α strains where lysE, dapA, dapB, asd, lysA, and ddh genes were introduced into the chromosome by the method described in Example 16. Randomly selected 200 strains were cultured in an SEII production medium containing 50 mg/L streptomycin and 20 mg/L kanamycin to select a strain which produced the most L-lysine, and was designated as Vmac1. A streptomycin-sensitive strain was selected from the Vmac1 strains to obtain a strain where pAET7 was eliminated. The plasmid pFLP31 shown in Example 9 was introduced into the strain, and a kanamycin-resistant gene region was eliminated from the mini-Mu cassette on the chromosome by the method described in Example 9. The kanamycin-sensitive strains obtained by the procedure were designated as Vmac1S strain. A streptomycin-sensitive strain was selected from Vmac1S strain to obtain a strain where the plasmid pFLP31 was eliminated. The plasmid pAET7 was introduced again into this strain, and the plasmid pMIV-FRTKmFRT-EABDAS#10 was also introduced again. Then, a strain which produced the most amount of L-lysine was selected and designated as Vmac2 strain. In the same way as described above, Vmac2S strain, Vmac3 strain, and Vmac3S strain were obtained. The amount of Lys produced by V12 was defined as 100, and the relative value of the amount of Lys produced by Vmac3S strain was calculated and shown in Table 10.

[0096]

[Table 10]

Name of strain	Relative amount of produced Lys (%)
V12	100
V12S α	100
Vmac3S	188

Example 19

<Impartation of L-Met auxotrophy by deletion of metF, construction of V3E2F strain, and evaluation of L-lysine productivity>

[0097] It was found that the amount of accumulated L-lysine was further increased by introducing the pBGEA plasmid into Vmac3S strain and that lysE24+dapA* was effective to increase the yield of L-lysine in Vmac3S strain, and therefore, a mini-Mu cassette of lysE24+dapA* was incorporated into chromosome again. Specifically, pAET7 was introduced into Vmac3S strain, and the plasmid pMIV-FRTKmFRT-EA was introduced thereto. The strain was cultured in an SEII agar medium containing 50 mg/L streptomycin and 20 mg/L kanamycin to select Vmac3S strains where lysE24+dapA* was incorporated in the chromosome. Randomly selected 200 strains were cultured in an SEII production medium containing 50 mg/L streptomycin and 20 mg/L kanamycin to select a strain which produced the most amount of L-lysine, and was designated as Vmac3EA. Transfer was repeated three times in accordance with completely the same method as in Example 3, and a strain which produced the most amount of L-lysine was selected and designated as V3E2 strain. An antibiotic-resistant marker was eliminated from the strain by the above-mentioned method to produce a kanamycin-sensitive strain, which was designated as V3E2S.

Next, L-methionine auxotrophy effective for improving L-lysine accumulation was impaired to VAE#8 strain. Specifically, three PCR-amplified DNA fragments designed by PCR so as to have overlapping regions were mixed, and the mixture was used as a template to perform PCR again, to thereby prepare a DNA fragment for gene disruption, where a kanamycin-resistant gene was inserted into nearly the center of the target gene. A fragment 1 (N-terminal side fragment) was amplified by PCR using a chromosomal DNA of *M.methylotrophus* AS1 strain as a template and using metF-fn1 (SEQ ID NO: 30: 5'-TGGAAGTACGGTGGCTACTC-3') and metF-rn (SEQ ID NO: 32: 5'-CCAGCCTACACAATCGCTCAAGACGTGTAAT-GCACTTCCGGATGAACTCAGGG TAAG-3') as primers. PCR was performed under the following conditions for 25 cycles: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension reaction at 72°C for 30 seconds. In the same way as described above, a fragment 2 (C-terminal side fragment) was amplified by PCR using a chromosomal DNA of *M.methylotrophus* AS 1 strain as a template and using metF-rc1 (SEQ ID NO: 33: 5'-TGCCAAATACGGGCTACTG-3') and metF-fc (SEQ ID NO: 35: 5'-GAGAATAGGAAGTTCGGAATAGGAAGTAAGGAG-GAGCTGGTTGCGTTTACGTC-3') as primers. PCR was performed under the following conditions for 25 cycles: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension reaction at 72°C for 30 seconds. A fragment 3 (Km-resistant gene fragment) amplified by PCR using the pKD4 plasmid as a template and using pKD-Kmf (SEQ ID NO: 36: 5'-GCATTACAGTCTTGAGCGATTGTGTAGGC-3') and pKD-KmGmr (SEQ ID NO: 37: 5'-CCTCCT-TAGTTCCTATTCCGAAGTTCCTATTCTC-3') as primers. PCR was performed under the following conditions for 25 cycles: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension reaction at 72°C for 30 seconds. The fragments 1 and 2 were separated in a 1% low-melting-point agarose gel and purified by Wizard PCR Preps DNA purification system manufactured by Promega. The concentrations of the fragments were measured, and PCR was performed using a mixture containing the fragments 1, 2, and 3 at a concentration ratio of 2 : 2 : 1 as a template and using metF-fn2 (SEQ ID NO: 31: 5'-GACCACGTCATTTCCCT-3') and metF-rc2 (SEQ ID NO: 34: 5'-TCCGGGCTCAAT-TCACTC-3') as primers. PCR was performed under the following conditions: a cycle consisting of denaturation at 98°C for 20 seconds and extension reaction at 68°C for 3 minutes was repeated for 25 cycles. In this amplification, LA taq polymerase (Takara Bio Inc.) was used. The amplified DNA fragment has a structure where a kanamycin-resistant gene is inserted into nearly the center of a gene fragment containing the metF gene. About 3 micrograms of the DNA fragment was prepared and introduced into V3E2S strain by electroporation. Recovery culture was performed in an SEII liquid medium containing 0.4 g/L L-methionine, and the cells were inoculated on an SEII agar medium containing 20 mg/L kanamycin, 0.4 g/L L-methionine, and 2.5 g/L sodium pyruvate. A strain, the colony of which that could not grow on an SEII agar medium containing no L-methionine, that is, a strain auxotrophic for L-methionine was selected and designated as V3E3F. When the strain was cultured in an SEII production medium containing 0.075 g/L L-methionine and 2.5 g/L sodium pyruvate and supplemented with ammonium sulfate to a final concentration of 6 g/L, the amount of produced Lys was increased. The amount of Lys produced by Vmac3S was defined as 100, and the relative value of the amount of Lys produced by V3E2F strain was calculated and shown in Table 11. The increase in the amount of produced L-lysine was thought to be caused by the decrease in the cell amount by imparting L-methionine auxotrophy. Table 12 shows the dapA activities of the wild-type strain and V3E2F strain.

[0098]

[Table 11]

Name of strain	Relative amount of produced Lys (%)
Vmac3S	100

(continued)

Name of strain	Relative amount of produced Lys (%)
V3E2	128
V3E2F	150

[0099]

[Table 12]

Name of bacterium	Specific activity (milliunit/milligram protein)
AS1	12
V3E2F	188

[0100] The amount of an enzyme that produces 1 micromol of a product per minute was defined as 1 unit.

Example 20

<Construction of plasmid pBG-lysC, construction of V3E2F/pBGlysC strain, and evaluation of L-lysine productivity>

[0101] Although the above-mentioned studies reveal that enhancement of aspartokinase activity in *M.methylotrophus* can improve L-lysine productivity, as shown in Example 15, enhancement of a wild-type AK derived from *M.methylotrophus* has been performed so far. However, the activity of an aspartokinase encoded by ask gene is subject to feedback inhibition by L-lysine and L-threonine. In order to improve L-lysine productivity, it is desirable that the activity is not subject to feedback inhibition by L-lysine and L-threonine. Therefore, a mutant AKIII which is an aspartokinase derived from *E. coli* and is not subject to feedback inhibition by L-lysine was used. A mutant lysC gene encoding a mutant AKIII that is desensitized to feedback inhibition by L-lysine was prepared by PCR using two oligonucleotide primers designed based on the known sequence and using the known plasmid RSFD80 (WO95/16042) as a template. Primers used were plysC-f (SEQ ID NO: 38: 5'-GAACCTGCAGGCCCTGACACGAGGTAGATTATGTC-3') and plysC-r (SEQ ID NO: 39: 5'-CTTTCGGCTAGAAGAGCGAGATGCAGATAAAAAAATTAAAGGCAATTATTCTCC G-3'), and PCR was performed under the following conditions for 25 cycles: denaturation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension reaction at 72°C for 90 seconds. The resulting product was digested with Sse8387I and SapI, and the resulting fragment was ligated to a fragment obtained by digesting the known plasmid pRStac (JP 2003-61687 A) with Sse8387I-SapI. The obtained plasmid was designated as pRSlysC*. Next, the pRSlysC* was digested with EcoRI-SapI to excise a lysC* fragment containing a tac promoter sequence, and the resulting product was ligated to a fragment obtained by digesting the known plasmid pBHR1 with the restriction enzyme DraI followed by blunt-ending and dephosphorylation. The plasmid was designated as pBHR-lysC plasmid. It was digested with NcoI, and the resulting fragment was blunt-ended and dephosphorylated to obtain a vector, into which the gentamicin-resistant gene fragment prepared in the same way as Example 11 was inserted to construct the plasmid pBGlysC. When the plasmid pBGlysC was introduced into V3E2F strain prepared in Example 19, the amount of produced L-lysine was increased. Moreover, when the known pRSlysEdapA (see JP 2003-61687 A) was introduced into the V3E2F/pBGlysC strain, the amount of produced L-lysine was increased. When the V3E2F/pBGlysC strain and V3E2F/pBGlysC/pRSlysEdapA strain were cultured in an SEII production medium containing 50 mg/L gentamicin, 50 mg/L streptomycin (the medium for the control strain contained neither gentamicin nor streptomycin), 0.08 g/L L-methionine, and 2.5 g/L sodium pyruvate and supplemented with ammonium sulfate at a final concentration of 6 g/L, the amount of produced Lys was increased. The amount of Lys produced by V3E2F was defined as 100, and the relative values of the amounts of Lys produced by V3E2F/pBGlysC and V3E2F/pBGlysC/pRSlysEdapA strains were calculated and shown in Table 13.

[0102]

[Table 13]

Name of strain	Relative amount of produced Lys (%)
V3E2F	100
V3E2F/pBGlysC	115
V3E2F/pBGlysC/pRSlysEdapA	132

Industrial Applicability

[0103] According to the present invention, L-lysine productivity of a methanol-assimilating bacterium is improved.

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	65 70 75 80	
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	130 135 140	
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	195 200 205	
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	210 215 220	
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Asp Leu Ala Asp Gly Arg Ile Pro Val Ile Ala Gly Thr Gly Ala Asn
65 70 75 80
Ala Thr Ala Glu Ala Ile Ser Leu Thr Gln Arg Phe Asn Asp Ser Gly
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210 215 220
His Phe Ala Glu Ala Arg Val Ile Asn Gln Arg Leu Met Pro Leu His
225 230 235 240
Asn Lys Leu Phe Val Glu Pro Asn Pro Ile Pro Val Lys Trp Ala Cys
245 250 255
Lys Glu Leu Gly Leu Val Ala Thr Asp Thr Leu Arg Leu Pro Met Thr
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Ala Thr Val Arg Ala Gly Asp Ile Val Gly Glu His Thr Ala Met Phe
210 215 220

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	Thr Gln Met Gly His Leu Tyr Gly His Val Ala Asp Glu Leu Ala Thr	
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	Pro Ser Ser Ala Ile Leu Asp Ile Glu Arg Lys Val Thr Thr Leu Thr	
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	ccg acc gtg gaa gaa ctg ctg gct gcg cac aat ccg tgg gcg aaa gtc	912
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	Val Arg Leu Val Val Leu Ser Ala Ser Ala Gly Ile Thr Asn Leu Leu	
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	Val Ala Leu Ala Glu Gly Leu Glu Pro Gly Glu Arg Phe Glu Lys Leu	
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	Asp Ala Ile Arg Asn Ile Gln Phe Ala Ile Leu Glu Arg Leu Arg Tyr	
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	Pro Asn Val Ile Arg Glu Glu Ile Glu Arg Leu Leu Glu Asn Ile Thr	
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	gtt ctg gca gaa gcg gcg gcg ctg gca acg tct ccg gcg ctg aca gat	336
	Val Leu Ala Glu Ala Ala Ala Leu Ala Thr Ser Pro Ala Leu Thr Asp	
	100 105 110	
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	Glu Leu Val Ser His Gly Glu Leu Met Ser Thr Leu Leu Phe Val Glu	
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	Ile Leu Arg Glu Arg Asp Val Gln Ala Gln Trp Phe Asp Val Arg Lys	
	130 135 140	
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	Val Met Arg Thr Asn Asp Arg Phe Gly Arg Ala Glu Pro Asp Ile Ala	
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	Gly Leu Val Ile Thr Gln Gly Phe Ile Gly Ser Glu Asn Lys Gly Arg	
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	Thr Thr Thr Leu Gly Arg Gly Gly Ser Asp Tyr Thr Ala Ala Leu Leu	
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	Asp Glu Ile Ala Phe Ala Glu Ala Ala Glu Met Ala Thr Phe Gly Ala			
	245	250	255	
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	Lys Val Leu His Pro Ala Thr Leu Leu Pro Ala Val Arg Ser Asp Ile			
	260	265	270	
	ccg gtc ttt gtc gcc tcc agc aaa gac cca cgc gca ggt ggt acg ctg			864
	Pro Val Phe Val Gly Ser Ser Lys Asp Pro Arg Ala Gly Gly Thr Leu			
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	gtg tgc aat aaa act gaa aat ccg ccg ctg ttc cgc gct ctg gcg ctt			912
	Val Cys Asn Lys Thr Glu Asn Pro Pro Leu Phe Arg Ala Leu Ala Leu			
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	Ser Arg Gly Phe Leu Ala Glu Val Phe Gly Ile Leu Ala Arg His Asn			
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	att tcg gta gac tta atc acc acg tca gaa gtg agc gtg gca tta acc			1056
	Ile Ser Val Asp Leu Ile Thr Thr Ser Glu Val Ser Val Ala Leu Thr			
	340	345	350	
	ctt gat acc acc ggt tca acc tcc act gcc gat acg ttg ctg acg caa			1104
30	Leu Asp Thr Thr Gly Ser Thr Ser Thr Gly Asp Thr Leu Leu Thr Gln			
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	tct ctg ctg atg gag ctt tcc gca ctg tgt cgg gtg gag gtg gaa gaa			1152
	Ser Leu Leu Met Glu Leu Ser Ala Leu Cys Arg Val Glu Val Glu Glu			
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	ggc gtt ggc aaa gag gta ttc gcc gta ctg gaa ccg ttc aac att cgc			1248
40	Gly Val Gly Lys Glu Val Phe Gly Val Leu Glu Pro Phe Asn Ile Arg			
	405	410	415	
	atg att tgt tat gcc gca tcc agc cat aac ctg tgc ttc ctg gtg ccc			1296
	Met Ile Cys Tyr Gly Ala Ser Ser His Asn Leu Cys Phe Leu Val Pro			
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 Pro Asn Val Ile Arg Glu Glu Ile Glu Arg Leu Leu Glu Asn Ile Thr
 85 90 95
 20 Val Leu Ala Glu Ala Ala Ala Leu Ala Thr Ser Pro Ala Leu Thr Asp
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 Glu Leu Val Ser His Gly Glu Leu Met Ser Thr Leu Leu Phe Val Glu
 115 120 125
 Ile Leu Arg Glu Arg Asp Val Gln Ala Gln Trp Phe Asp Val Arg Lys
 130 135 140
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 Ala Leu Ala Glu Leu Ala Ala Leu Gln Leu Leu Pro Arg Leu Asn Glu
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 Thr Thr Thr Leu Gly Arg Gly Gly Ser Asp Tyr Thr Ala Ala Leu Leu
 195 200 205
 35 Ala Glu Ala Leu His Ala Ser Arg Val Asp Ile Trp Thr Asp Val Pro
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 Gly Ile Tyr Thr Thr Asp Pro Arg Val Val Ser Ala Ala Lys Arg Ile
 225 230 235 240
 Asp Glu Ile Ala Phe Ala Glu Ala Ala Glu Met Ala Thr Phe Gly Ala
 245 250 255
 40 Lys Val Leu His Pro Ala Thr Leu Leu Pro Ala Val Arg Ser Asp Ile
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 Arg Arg Asn Gln Thr Leu Leu Thr Leu His Ser Leu Asn Met Leu His
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 325 330 335
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 55 Leu Asp Thr Thr Gly Ser Thr Ser Thr Gly Asp Thr Leu Leu Thr Gln
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Ser Leu Leu Met Glu Leu Ser Ala Leu Cys Arg Val Glu Val Glu Glu
 370 375 380
 Gly Leu Ala Leu Val Ala Leu Ile Gly Asn Asp Leu Ser Lys Ala Cys
 5 385 390 395 400
 Gly Val Gly Lys Glu Val Phe Gly Val Leu Glu Pro Phe Asn Ile Arg
 405 410 415
 Met Ile Cys Tyr Gly Ala Ser Ser His Asn Leu Cys Phe Leu Val Pro
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 Ala Leu Arg Asp Ile Ala Gln Thr His Gln Thr Pro Thr Tyr Val Tyr
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 Ser Arg Ala Ala Leu Thr Thr Ala Phe Glu Arg Phe Gln Ala Gly Leu
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 act gga cat gac cat ttg atc tgc ttt gct gtc aaa gcc aac cca agc 192
 40 Thr Gly His Asp His Leu Ile Cys Phe Ala Val Lys Ala Asn Pro Ser
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 ctg gcc att ctc aac ctg ttt gcg cga atg gga gcg ggc ttt gat att 240
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 45 gtg tcc ggt ggt gag ctg gca cgc gtc ttg gcc gca ggt ggc gac ccg 288
 Val Ser Gly Gly Glu Leu Ala Arg Val Leu Ala Ala Gly Gly Asp Pro
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 aaa aaa gtg gtg ttt tct ggt gtg ggc aaa tcc cat gcg gaa atc aaa 336
 50 Lys Lys Val Val Phe Ser Gly Val Gly Lys Ser His Ala Glu Ile Lys
 100 105 110
 gcc gcg ctt gaa gcg ggc att ctt tgc ttc aac gtg gaa tca gtg aat 384
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	Tyr	Ile	Ser	Thr	Gly	Leu	Lys	Asn	Asn	Lys	Phe	Gly	Val	Ala	Phe	Glu	
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	Ala	Ala	Lys	Gly	Ile	His	Ile	Gln	His	Ile	Asp	Val	Gly	Gly	Gly	Val	
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	Glu	Pro	Gly	Arg	Ala	Leu	Val	Gly	Asn	Ala	Gly	Val	Leu	Leu	Thr	Lys	
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35	Val	Glu	Tyr	Leu	Lys	Pro	Gly	Glu	Thr	Lys	Asn	Phe	Ala	Ile	Val	Asp	
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	Ala	Ala	Met	Asn	Asp	Leu	Met	Arg	Pro	Ala	Leu	Tyr	Asp	Ala	Phe	His	
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	Ile	Val	Gly	Pro	Val	Cys	Glu	Ser	Gly	Asp	Phe	Leu	Gly	His	Asp	Arg	
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	Thr	Leu	Ala	Ile	Glu	Glu	Gly	Asp	Tyr	Leu	Ala	Ile	His	Ser	Ala	Gly	
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Thr Gly His Asp His Leu Ile Cys Phe Ala Val Lys Ala Asn Pro Ser
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Leu Ala Ile Leu Asn Leu Phe Ala Arg Met Gly Ala Gly Phe Asp Ile
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Val Ser Gly Gly Glu Leu Ala Arg Val Leu Ala Ala Gly Gly Asp Pro
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Lys Lys Val Val Phe Ser Gly Val Gly Lys Ser His Ala Glu Ile Lys
 100 105 110

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Ala Ala Leu Glu Ala Gly Ile Leu Cys Phe Asn Val Glu Ser Val Asn
 115 120 125

Glu Leu Asp Arg Ile Gln Gln Val Ala Ala Ser Leu Gly Lys Lys Ala
 130 135 140

Pro Ile Ser Leu Arg Val Asn Pro Asn Val Asp Ala Lys Thr His Pro
 145 150 155 160

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Tyr Ile Ser Thr Gly Leu Lys Asn Asn Lys Phe Gly Val Ala Phe Glu
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Asp Ala Leu Gly Leu Tyr Glu Lys Ala Ala Gln Leu Pro Asn Ile Glu
 180 185 190

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Val His Gly Val Asp Cys His Ile Gly Ser Gln Ile Thr Glu Leu Ser
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Pro Phe Leu Asp Ala Leu Asp Lys Val Leu Gly Leu Val Asp Ala Leu
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Ala Ala Lys Gly Ile His Ile Gln His Ile Asp Val Gly Gly Gly Val
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Gly Ile Thr Tyr Ser Asp Glu Thr Pro Pro Asp Phe Ala Ala Tyr Thr
 245 250 255

Ala Ala Ile Leu Lys Lys Leu Ala Gly Arg Asn Val Lys Val Leu Phe
 260 265 270

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Glu Pro Gly Arg Ala Leu Val Gly Asn Ala Gly Val Leu Leu Thr Lys
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Val Glu Tyr Leu Lys Pro Gly Glu Thr Lys Asn Phe Ala Ile Val Asp
 290 295 300

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Ala Ala Met Asn Asp Leu Met Arg Pro Ala Leu Tyr Asp Ala Phe His

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	Ile	Lys	Arg	Glu	Gly	Leu	Ile	Ala	Val	Leu	Leu	Val	Cys	Leu	Ile	Ser	
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	Tyr	Leu	Leu	Trp	Phe	Ala	Val	Met	Ala	Ala	Lys	Asp	Ala	Met	Thr	Asn	
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	Cys Met Gly Ser Ala Thr Asp Ile Pro Glu Gln Ala Pro Lys Phe Ala			
	65	70	75	80
10	cag ttc gcc tgc acc gta gac acc tac gac aac cac cgc gac atc cca	288		
	Gln Phe Ala Cys Thr Val Asp Thr Tyr Asp Asn His Arg Asp Ile Pro			
	85	90	95	
	cgc cac cgc cag gtc atg aac gaa gcc gcc acc gca gcc ggc aac gtt	336		
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	gca ctg gtc tct acc ggc tgg gat cca gga atg ttc tcc atc aac cgc	384		
	Ala Leu Val Ser Thr Gly Trp Asp Pro Gly Met Phe Ser Ile Asn Arg			
	115	120	125	
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Claims

1. A methanol-assimilating bacterium, which comprises:

a DNA encoding dihydrodipicolinate synthase which is desensitized to feedback inhibition by L-lysine; and
a DNA encoding a mutant LysE protein that promotes export of L-lysine to the outside of a bacterial cell when
it is introduced into a methanol-assimilating bacterium, wherein said bacterium is modified to enhance the
intracellular activities of diaminopimelate dehydrogenase, diaminopimelate decarboxylase, dihydrodipicolinate
reductase, and aspartate-semialdehyde dehydrogenase.

2. The methanol-assimilating bacterium according to claim 1, further comprising:

a DNA encoding an aspartokinase which is desensitized to feedback inhibition by L-lysine.

3. The methanol-assimilating bacterium according to claim 1 or 2, wherein said bacterium is introduced with DNAs
encoding the enzymes or the mutant LysE protein into chromosomal DNA and/or transformed with a plasmid(s)
carrying DNAs encoding the enzymes or the mutant LysE protein.

4. The methanol-assimilating bacterium according to claim 3, wherein the DNA encoding dihydrodipicolinate synthase,
the DNA encoding dihydrodipicolinate reductase, the DNA encoding aspartate-semialdehyde dehydrogenase, and
the DNA encoding aspartokinase are derived from a bacterium belonging to the genus *Escherichia*, the DNA encoding
diaminopimelate decarboxylase is derived from a bacterium belonging to the genus *Methylophilus*, and the DNA
encoding diaminopimelate dehydrogenase and the DNA encoding mutant LysE are derived from a bacterium be-
longing to the genus *Brevibacterium*.

5. The methanol-assimilating bacterium according to any one of claims 1 to 4, wherein said bacterium belongs to the
genus *Methylophilus*.

6. The methanol-assimilating bacterium according to any one of claims 1 to 5, wherein said bacterium is *Methylophilus
methylotrophus* AJ110196 strain (FERM BP-10434).

7. A method of producing L-lysine, comprising culturing the methanol-assimilating bacterium according to any one of
claims 1 to 6 in a medium to produce and accumulate L-lysine in the medium or bacterial cells; and collecting L-
lysine from the medium or bacterial cells.

8. The method of producing L-lysine according to claim 7, wherein the main carbon source of the medium is methanol.

9. *Methylophilus methylotrophus* AJ110196 strain (FERM BP-10434).

Fig. 1

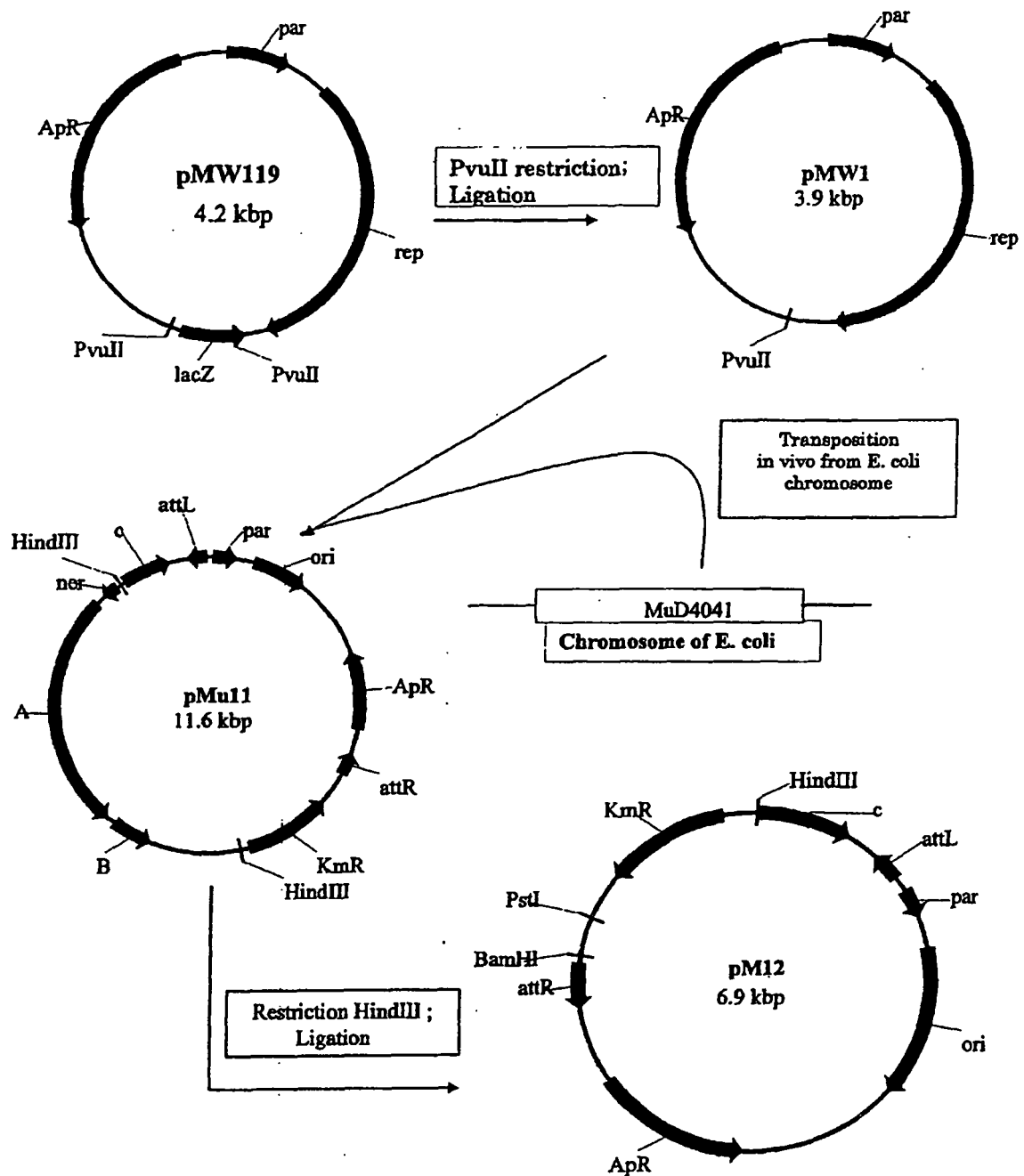


Fig. 2

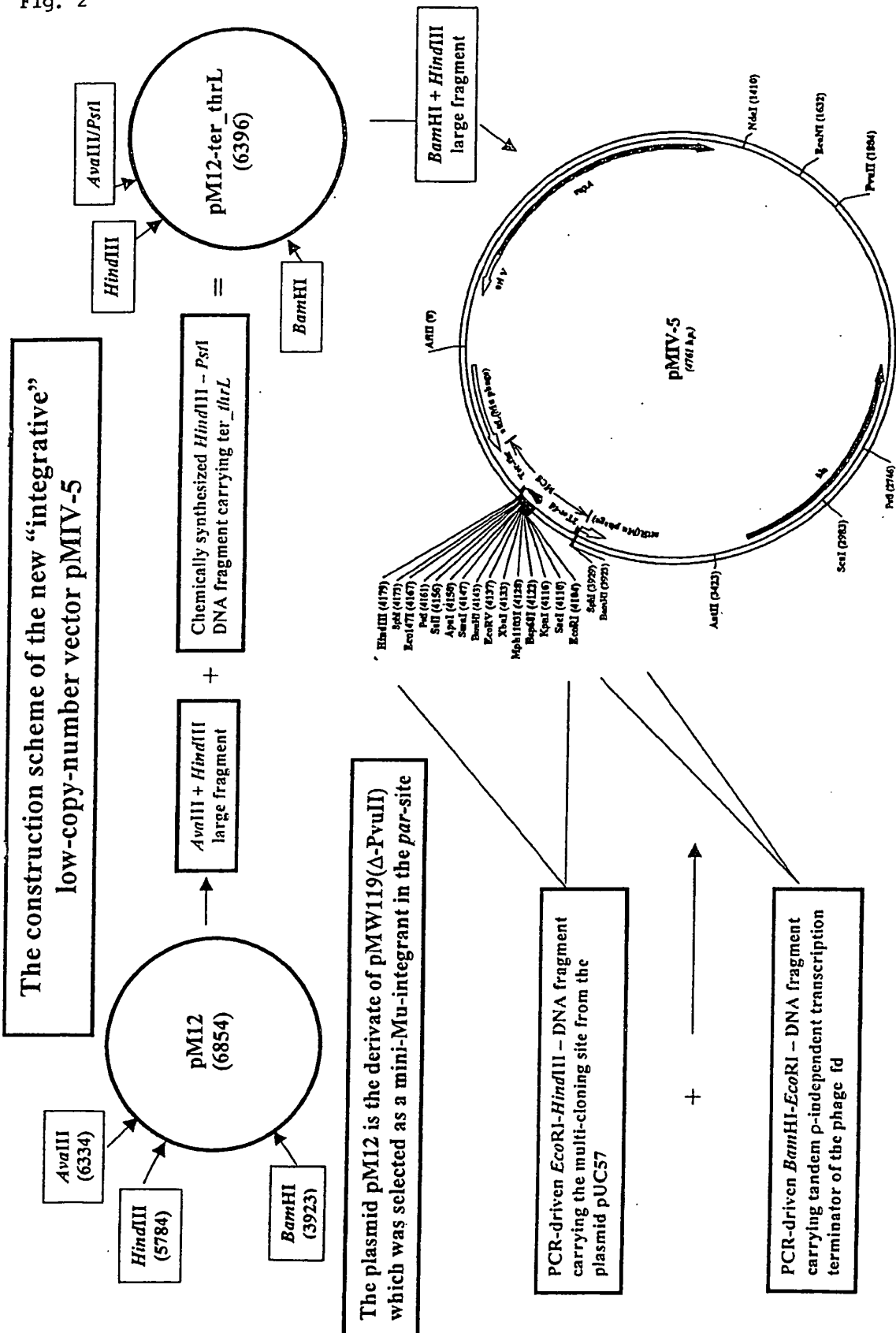


Fig. 3

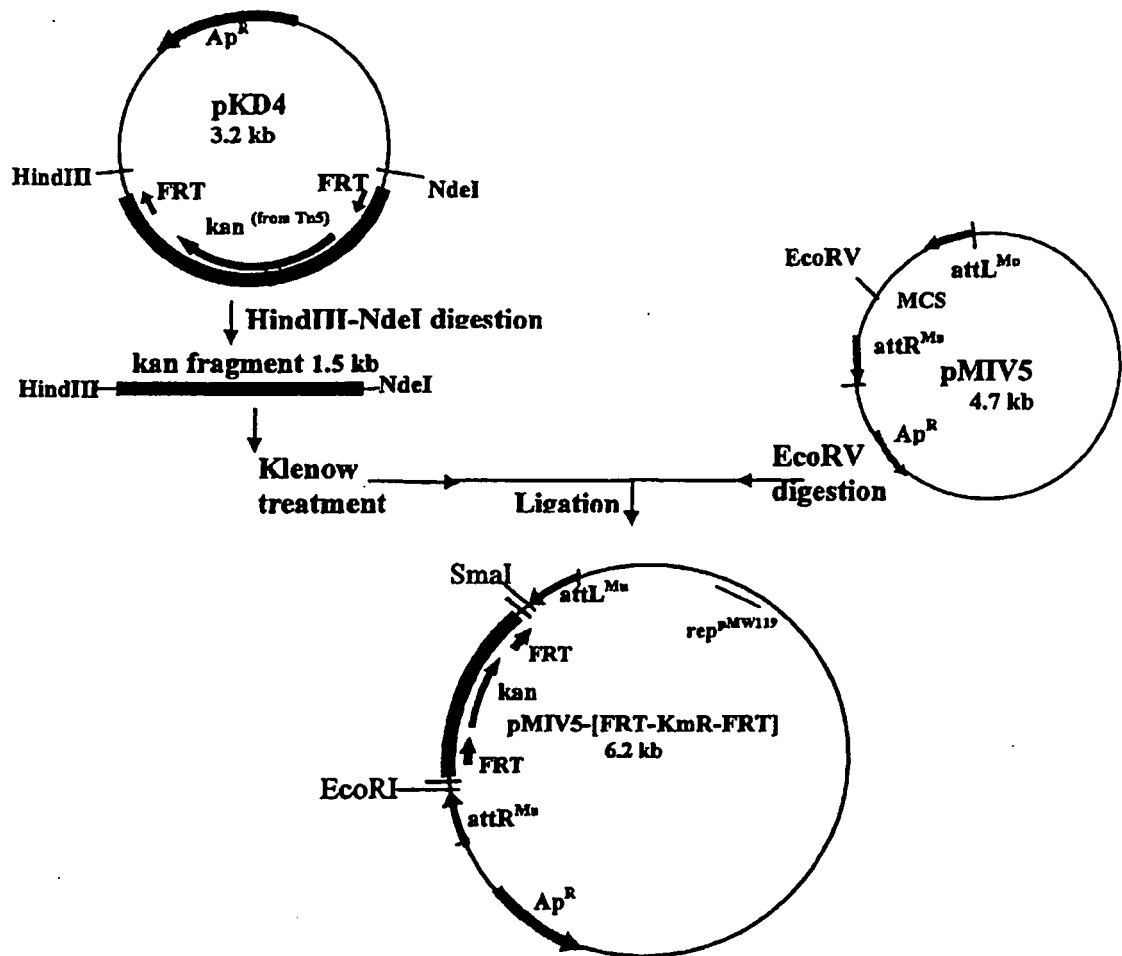


Fig. 4

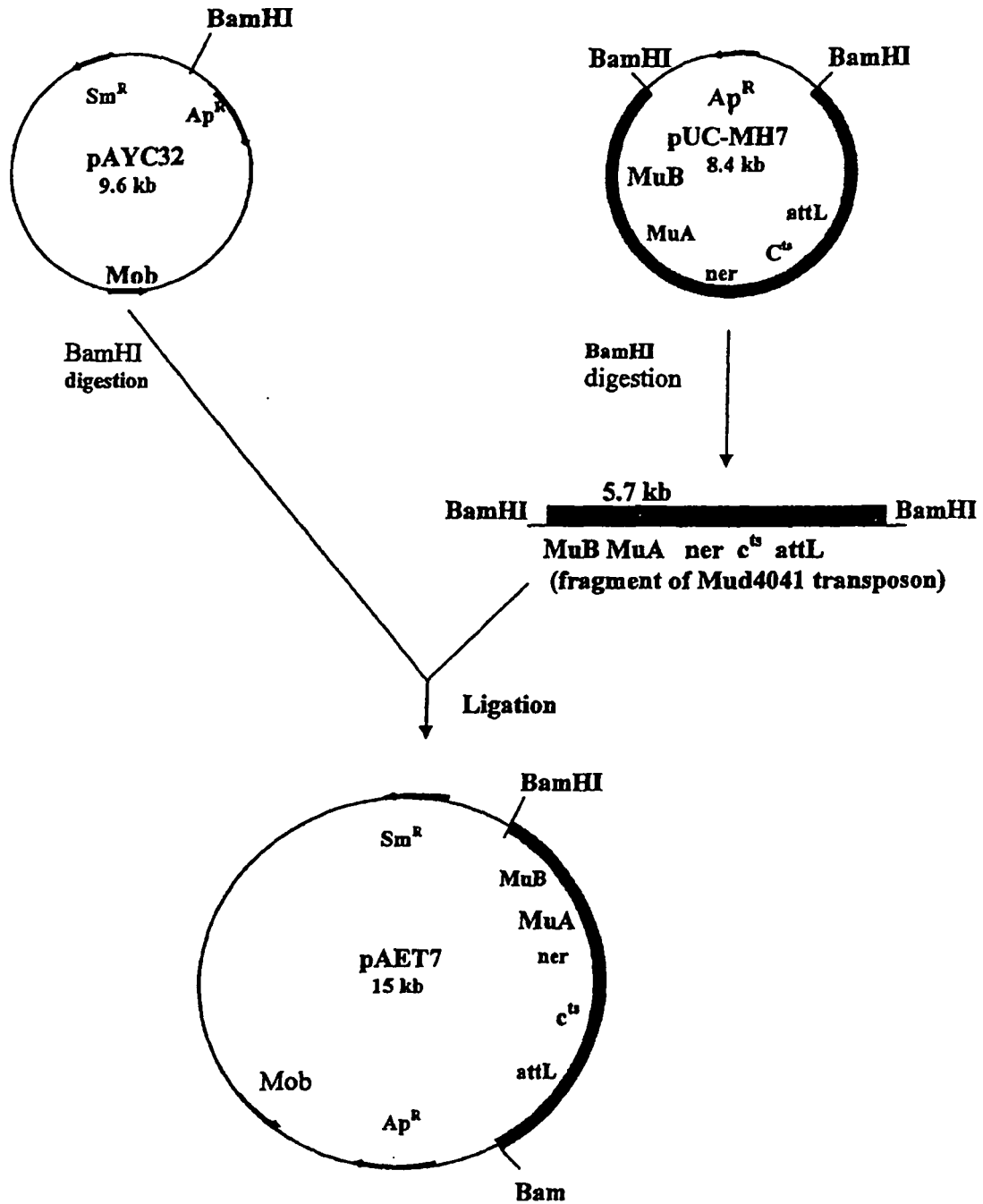
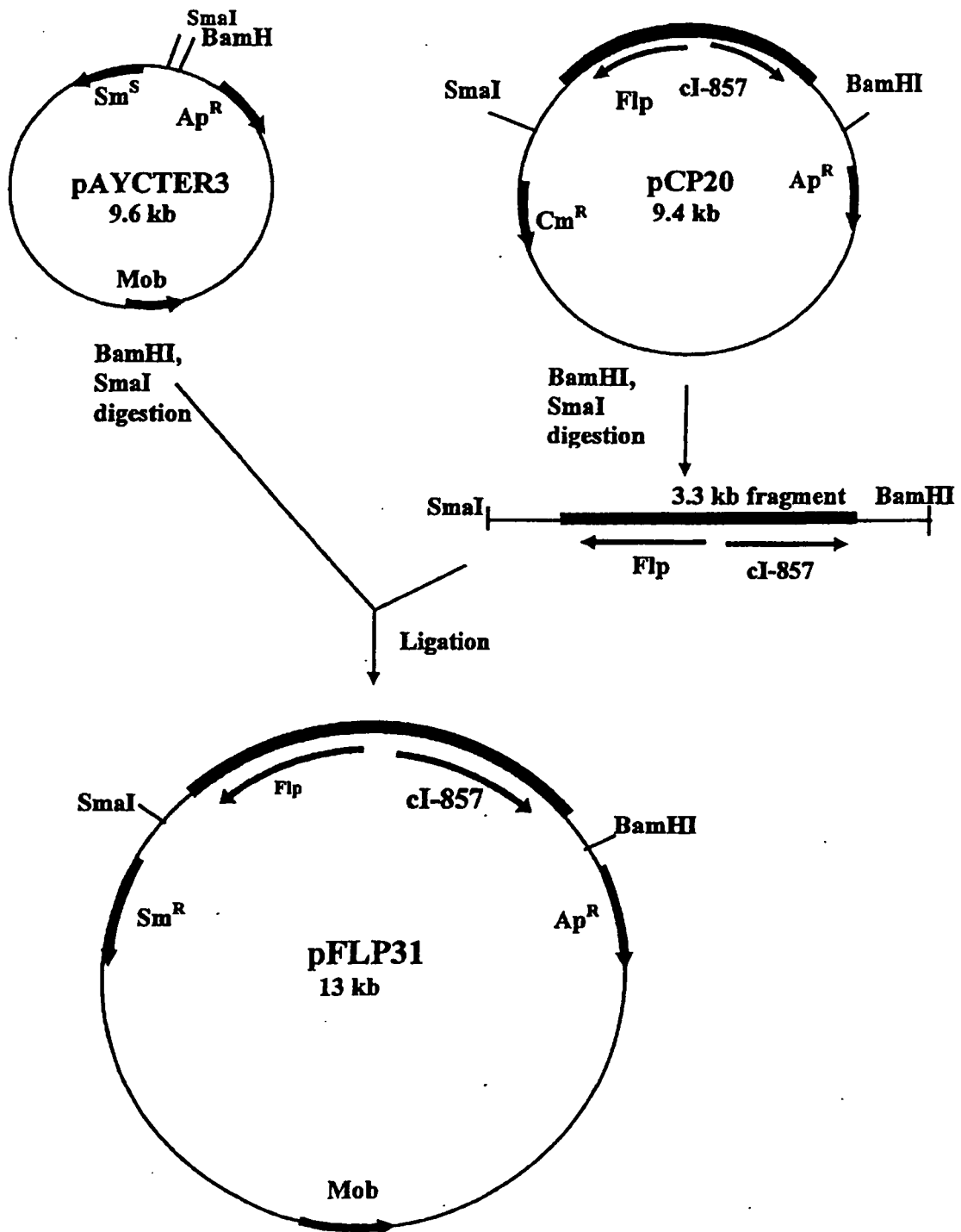


Fig. 5



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2007/051796

A. CLASSIFICATION OF SUBJECT MATTER

C12N1/21(2006.01)i, C12N15/09(2006.01)i, C12P13/08(2006.01)i, C12R1/01(2006.01)n

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N1/21, C12N15/09, C12P13/08, C12R1/01

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS/MEDLINE/WPIDS/REGISTRY/Caplus (STN),
JMEDPlus/JST7580/JSTPlus (JDream2), GenBank/EMBL/DDBJ/GeneSeq,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP 2003-061687 A (Ajinomoto Co., Inc.), 04 March, 2003 (04.03.03), Par. Nos. [0031] to [0033], [0046], [0073] to [0076], [0092] to [0097] & EP 1266966 A & US 2003/0124687 A	1-9
Y	YOSHIYA G., et al., Characterization of the L-lysine biosynthetic pathway in the obligate methylophilic Methylophilus methylotrophus., Bioscience Biotechnology and Biochemistry, 2004, vol. 68, no. 7, p. 1449-1460	1-9
Y	WO 2000/061723 A (Ajinomoto Co., Inc.), 19 October, 2000 (19.10.00), Claim 9; page 12; example 1; table 2; example 7(5) & EP 1188822 B	1-9

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
23 February, 2007 (23.02.07)Date of mailing of the international search report
06 March, 2007 (06.03.07)Name and mailing address of the ISA/
Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2007/051796

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

SwissProt/PIR/Geneseq

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